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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 PNL, 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 Iysozyme. Recent publications have demonstrated the advantages of using alternating hydrostatic pressure (Pressure Cycling Technology, PCT) for proteomic sample preparation. We have developed and evaluated a novel sample preparation scheme, in which PCT is used to enhance cell lysis, protein extraction and proteolytic digestion, which are carried out in a single, chemically inert disposable sample container (PCT MicroTube).

The mechanisms of pressure-induced acceleration remain speculative, while the influence of various chemical agents on enzymatic activity, substrate conformation and efficiency of the digestion process are not well understood, which leads to great variability of experimental results. 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 in a variety of applications, including cell and tissue lysis, and the extraction of proteins, lipids and nucleic acids [8]. Recently, PCT has also been shown to accelerate enzymatic reactions such as proteolysis [1]. The PCT SPS (Figure 1) is comprised of a small, semi-automated benchtop instrument (Barocycler NEP3229 or NEP220) and single-use sample processing containers called 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.

In this work we explore the stability and catalytic activity of trypsin and chymotrypsin under the influence of several chaotropes and organic solvents in combination with hydrostatic pressure and elevated temperatures. We have employed chromogenic substrates in order to measure enzyme activity independent of pressure-induced changes in 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.



Figure 1. The PCT SPS platform

## 2. Materials and Methods

### 2.1 PCT MicroTube Sample Containers

In order to address the strong demand for smaller sample volumes and to enable higher sample throughput, new disposable processing containers (PCT 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 adsorption, broad solvent and temperature compatibility and minimal extractability. Several versions of the PCT MicroTube caps are available that allow the use of the same size PCT MicroTube with multiple sample volumes (50, 100 and 150 µl). These new containers are suitable for cell lysis, tissue lysis, PCT-fractionation as well as in-solution and in-gel protein digestion applications. The specialized cartridge system is designed to hold multiple (up to 48) PCT MicroTubes in the pressure chamber of the Barocycler instrument. The PCT MicroTube cartridge system keeps these containers sealed during rapid cycles of hydrostatic pressure exceeding the boiling point of the sample components. Additionally, the PCT MicroTube cap is designed to be used as a gel spot picking tool (Figure 2) - this approach reduces the likelihood of cross-contamination between gel spots and substantially simplifies the gel spot picking and transfer process. PCT MicroTubes withstand centrifugal forces up to 14,000 x g thus enabling stepwise fractionation of cell lysates by the reagents of increasing stringency directly in a single container.



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.

## 2.2 Enzyme Activity Assays

**Trypsin Activity:** Trypsin activity was measured using a chromogenic substrate, N-Benzoyl-DL-arginine 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 trypsin/BAPNA reaction mixture were subjected to pressure cycling at 37°C or 55°C. Pressure cycling was performed at the indicated pressure for 20 cycles. 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 controls incubated at the same temperature and pressure without denaturant. For all samples, results were read at 405 nm.

**α-Chymotrypsin Activity:** 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 containing 0.15 ml of chymotrypsin/substrate 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/l mixture of standard proteins with varying molecular weights, isoelectric points, and number of amino acid residues [Table 1] was used for examination of various digestion protocols. Each protocol used 5 pmol of protein and was done in triplicate. Samples were digested either in an incubated shaker at atmospheric pressure or by pressure cycling at 35,000 psi, 37°C, no HFIP. Results are expressed relative to control (35,000 psi, 37°C, no HFIP). The data indicate that at concentrations below 6%, HFIP leads to an increase of trypsin activity of ~20% above control. 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%.

## 2.4 Digestion of Whole Cell Proteome

HepG2 cells were grown in MEM with 10% FBS in several separate 10-cm dishes to 80% confluence. The cells in each plate were washed with PBS and harvested separately. 1.1,1.1,3.3 - hexafluoro-2-propanol (HFIP), an organic solvent, was added to some of the resulting cell suspension aliquots to the concentration of 30%. Other plates were harvested in aqueous buffer. Different methods of cell lysis and trypsin digestion were evaluated as indicated in Figure 12. Pressure cycling was used to simultaneously homogenize the sample, to facilitate the dissolution of cells, micelles and membrane fragments, and to increase the efficiency of hydrophobic protein recovery. After digestion and analysis by LC/MS, the number and properties of proteins identified in each lysate were determined and compared.

Protein Description	SwissProt accession #	MW (Da)	PI	AAs
Liquorin human	P62988	9382	7.30	82
Muoglobulin equine	P68082	16941	7.36	154
Cytochrome C bovine	P62894	11565	9.52	104
β-Casovin bovine	P02666	23568	5.13	209
Bovine serum albumin	P02769	66390	5.60	583
κ1-Casovin	P02662	22960	4.91	199
κ2-Casovin	P02663	24333	8.34	207
ε-Casovin	P02668	18963	5.93	169

Table 1. Standard protein mixture.

## 3. Results and Discussion

### 3.1 Enzymatic activity

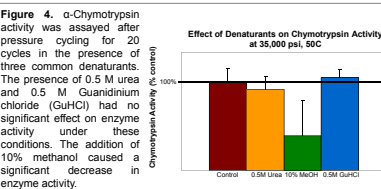
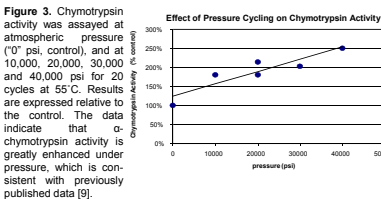


Figure 4. α-Chymotrypsin activity was assayed after pressure cycling for 20 cycles in the presence of three common denaturants. The presence of 0.5 M urea and 0.5 M Guanidium chloride (GuHCl) had no significant effect on enzyme activity under these conditions. The addition of 10% methanol caused a significant decrease in enzyme activity.

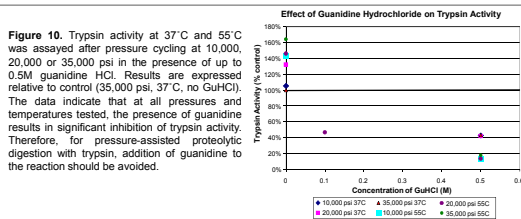
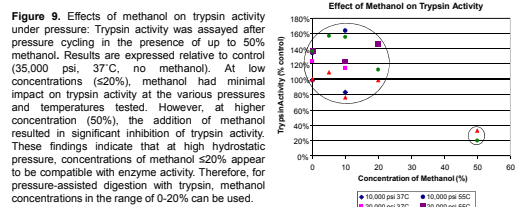
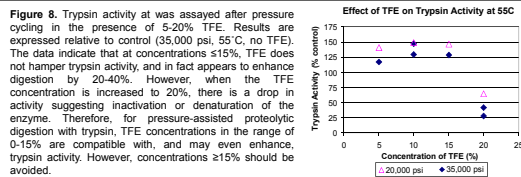
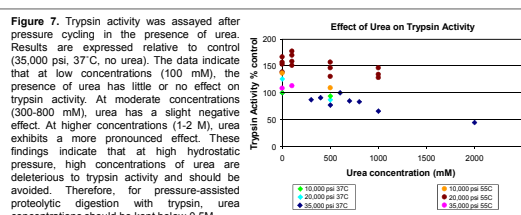
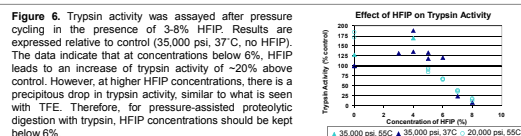
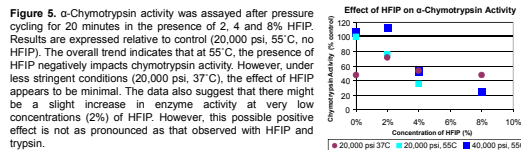


Figure 10. Trypsin activity was assayed after pressure cycling at 37°C and 55°C was assayed after pressure cycling at 10,000, 20,000 or 35,000 psi in the presence of up to 0.5M guanidine-HCl. Results are expressed relative to control (35,000 psi, 37°C, no GuHCl). The data indicate that at all pressures 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 and Cell Lysis.

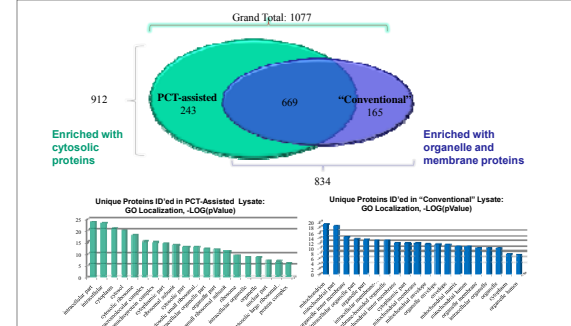
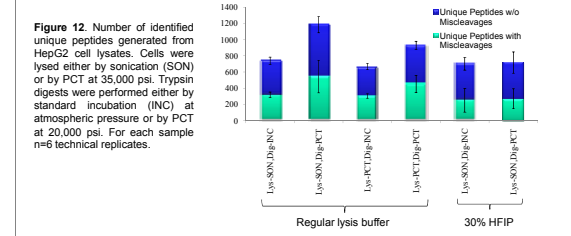
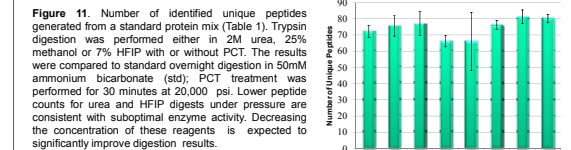


Figure 13. Overlap of detected HepG2 proteomes extracted by a conventional method (using sonication) and by PCT, all in aqueous buffer. The lower plots demonstrate GO localization terms differentially enriched in the non-overlapping fraction of detected proteomes.

## 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, trifluoromethanol (TFE) 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 sharply inhibitory at higher concentrations.
7. Pressure cycling can be used to enhance cell lysis and accelerate trypsin digestion in the same sample container while minimizing sample handling and potential loss of analytes.

## 5. References

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