Optimization of high pressure-assisted methods for proteomic sample preparation.

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Introduction

Rapid progress in mass spectrometry instrumentation development has enabled proteomics as a thriving research field. However, in spite of availability of powerful analytical tools, traditional methods of sample preparation continue to hamper the rates of protein biomarker discovery.

Hydrostatic pressure is known to acts synergistically with organic solvents, chaotropes and detergents in its effects on protein conformation and enzyme activity [1-5]. This phenomenon can be utilized to facilitate cell lysis, extraction and dissolution of hydrophobic membrane proteins and control proteolytic digestion of proteins for mass spectrometry-based proteomics. This report summarizes the use of hydrostatic pressure as a thermodynamic parameter orthogonal to temperature to control cell lysis, protein solubility and enzymatic activity in proteomic sample preparation. Since proteases are proteins themselves, we explored their activity under pressure in the presence of several chaotropes and organic solvents, used to promote denaturation of sample proteins.

Materials and Methods

Pressure Cycling Technology (PCT)

The Pressure Cycling Technology Sample Preparation Systems (PCT SPS, Figure 1) applies alternating hydrostatic pressure between atmospheric and up to 310 MPa to control molecular interactions [6]. The PCT SPS has been successfully used for cell and tissue lysis, extraction of proteins, lipids and nucleic acids [7,8]. Recently, PCT has been shown to accelerate enzymatic reactions such as proteolysis [9]. The PCT SPS is comprised of a semi-automated benchtop instrument (Barocycler NEP3229 or NEP2320) and single-use sample processing containers, allowing processing multiple samples in parallel.

Figure 1. The PCT SPS product line and accessories

PCT Sample Containers

PULSE Tubes FT500 and FT500-ND accommodate sample volumes from 200 to 1400 µL and pass the pressure to the sample via the freely moving ram on one end. The FT500 model also contains a "lysis disk" designed for tissue lysis. The PCT MicroTubes meet the requirements of proteomic analysis by mass spectrometry and synthetic chemistry applications. These containers are made of inert fluorinated ethylene propylene (FEP), have no moving parts and efficiently transmit hydrostatic pressure to the sample by flexible deformation of the polymer walls. The PCT MicroTubes retain the seal integrity within a wide temperature range (-200°C to +100°C). The cartridge system permits pressure cycling and incubation at temperatures above boiling point. Up to 48 MicroTubes can be processed simultaneously in a Barocycler NEP3229. The PCT MicroTube's outstanding chemical resistance, non-wetting surface, and minimal protein and nucleic acid binding, help to ensure nearly complete sample recovery. Specialized tool is provided for easy capping and decapping of the MicroTubes (Figure 2).

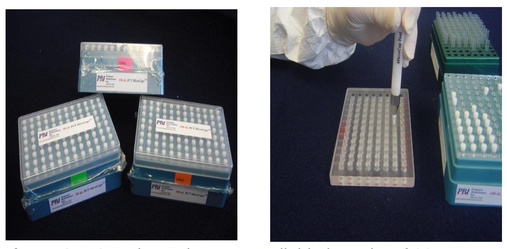

Figure 2. PCT MicroTubes are available in racks of 96.

Enzyme Activity Assays

Trypsin activity was measured using a chromogenic substrate, N_{α}-Benzoyl-D,L-argenine 4-nitroanilide hydrochloride (BAPNA). Assay was performed in 50 mM ammonium bicarbonate using 2 µg/ml trypsin (Promega) and 500 µg/ml BAPNA (Sigma). PCT MicroTubes containing 0.15 ml of reaction mixture were subjected to pressure cycling at 37˚C or 55˚C. Each pressure cycle consisted of 55 seconds at the indicated high pressure and 5 seconds at atmospheric pressure. To determine the effect of various denaturants 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, UV absorbance were read at 405nm 20 minutes after the reaction start.

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.

Pressure-Enhanced Enzymatic Proteolysis

Protein Extraction

Total protein was extracted from small biopsy-size rat tissue samples various rat tissues using a scaled-down version of the ProteoSolve-SB (Pressure BioSciences, Inc.) protocol. 10-20 mg of tissue per MicroTube was processed by PCT in 100 µL Reagent A + 35 µl Reagent B using the 150 µL PCT MicroCaps. PCT was performed at 35,000 psi for 20 cycles. SDS-PAGE was performed as described below (Figure 3).

In-Solution Protein Digestion

A 1 pmol/µL mixture of standard proteins 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 NEP3229 (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.

Table 1. Standard Protein Mixture

Protein Description	SwissProt #	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
β –Casein bovine	P02666	23568	5.13	209
Bovine serum albumin	P02769	66390	5.60	583
α1- Casein (bov.)	P02662	22960	4.91	199
α2- Casein (bov.)	P02663	24333	8.34	207
κ –Casein (bov.)	P02668	18963	5.93	169

In-Gel Protein Digestion

A mixture of the six proteins (Figure 6) was prepared containing 400 µg/mL of each protein. Six serial dilutions of this solution were mixed with NuPAGE LDS Sample Buffer, heat denatured, and loaded in triplicate on NuPAGE 4-12% Bis-Tris gels at 4, 2, 1, 0.5, 0.25, and 0.125 ug of each protein per lane. After electrophoretic separation the gels were stained with EZ Blue Gel Stain. All gel supplies were from Invitrogen (Carlsbad, CA).

Protein bands were excised into 0.6 mL Eppendorf tubes, destained in alternating 50 mM ABC / acetonitrile 4 times for 20 minutes each, reduced in 10 mM DTT and alkylated in 50 mM iodoacetamide, dried by SpeedVac and transferred to PCT MicroTubes or left for digestion directly in Eppendorf tubes. Proteins were digested with trypsin at enzyme:protein ratio of 1:20 using PCT for 45 cycles at 37 ºC, 35,000 psi, 55s up, 5s down (PCT-based digestion) or for 16 h. at 37ºC (control). Peptides were extracted with 50% acetonitrile and spiked with a mixture of five synthetic catalase peptides as internal standard at 50 fmol per 0.7 uL. A 0.7 µL aliquot of each sample was spotted on a MALDI target in triplicate followed by addition of CHCA matrix on each spot.

Mass spectra were acquired on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA) in positive reflectron mode. The resulting data were processed and interpreted using GPS Explorer™ version 3.6. MASCOT was used for database searching against SwisProt database, considering no missed cleavage and up to one, two or three missed cleavage(s), carbamidomethyl modifications of cysteine, monoisotopic peptide mass tolerance of 50 ppm, and fragment ion mass tolerance of 0.3 amu. The average peak intensities of the three most intense tryptic peptides for each protein were calculated and these values were normalized by the average peak intensity of the three most intense peptides of the internal standard.

Results and Discussion

Protein Extraction with PCT MicroTubes and ProteoSolve-SB

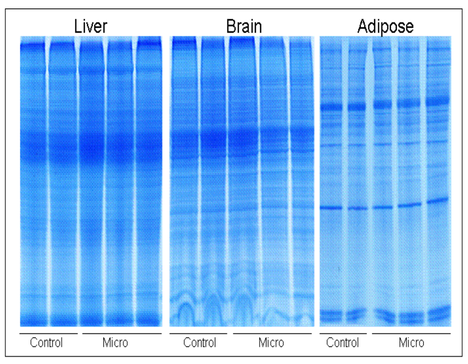

Figure 3. Reproducible protein recovery between sample replicas is demonstrated by SDS-PAGE. Samples are extracted into a detergent-free solvent system easily removed by evaporation after removal of nucleic acids and lipid fractions.

Enzymatic Activity

Chaotropic effect of fluorinated alcohols, urea and guanidine on trypsin and α-chymotrypsin activity **showed significant boost under pressure.** 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. Therefore, for pressure-assisted proteolytic digestion with trypsin, HFIP concentrations should be kept below 6%. The same trend is observed for chymotrypsin at lower concentrations (2%) of HFIP but is less pronounced. Trypsin, but not chymotrypsin activity benefits from addition of moderate amounts of trifluoroethanol. Presence of guanidine above 0.1M have a very pronounced negative effect on trypsin activity, but not affecting chymotrypsin as much (data not shown). Urea below 2M has moderate inhibitory effect on trypsin at a range of temperature and pressure conditions, although it is less desirable due to the potential of protein carbamylation at high temperature.

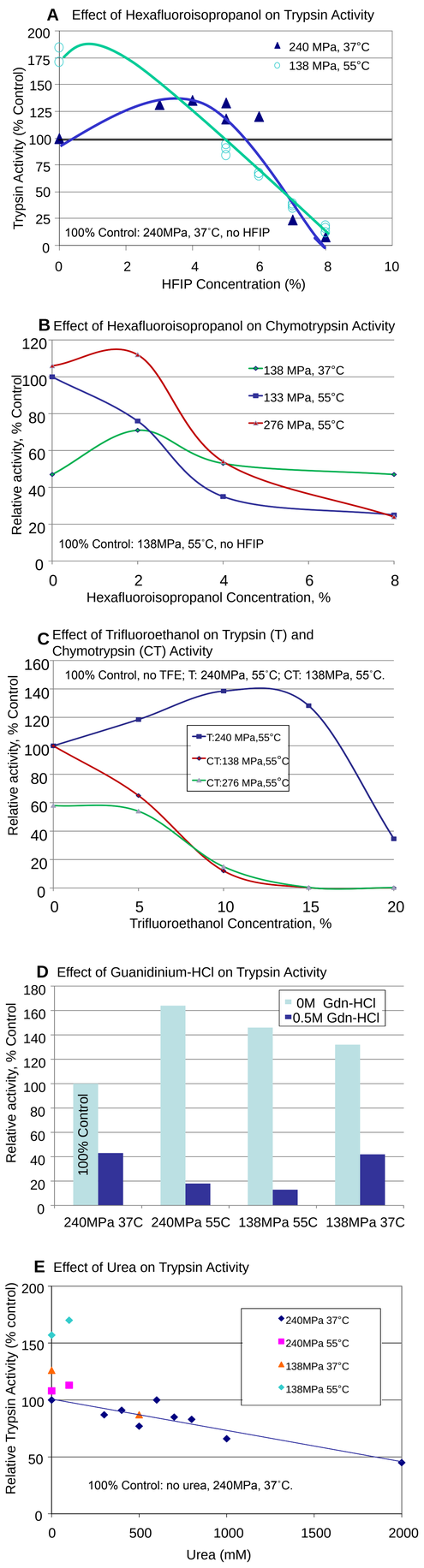

Figure 5 A-E. Enzymatic activity under pressure

In-Solution Protein Digestion

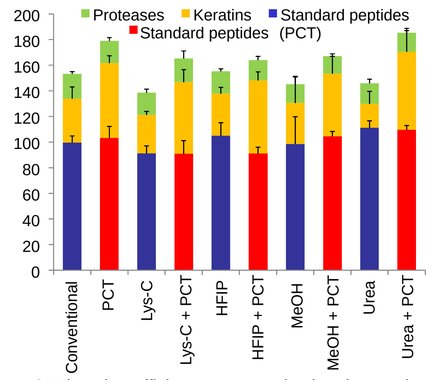

Figure 6. Digestion efficiency assessed using the number of identified unique peptides in Protein Mixture using Eppendorf and PULSE tubes (n=6).

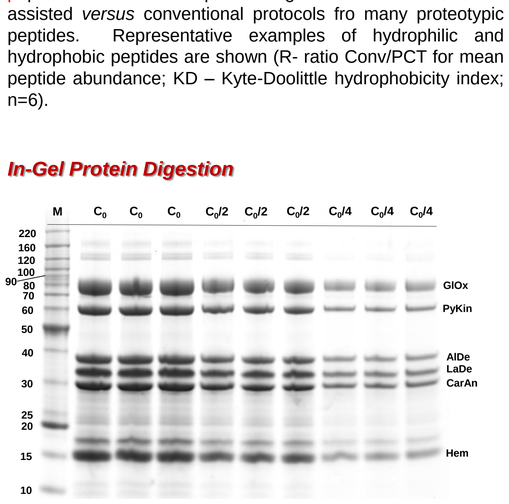

Figure 7. Quantitative analysis of peptide recovery. Higher peptide abundance in protein digests resulted from PCT-assisted *versus* conventional protocols fro many proteotypic peptides. Representative examples of hydrophilic and hydrophobic peptides are shown (R- ratio Conv/PCT for mean peptide abundance; KD – Kyte-Doolittle hydrophobicity index; n=6).

In-Gel Protein Digestion

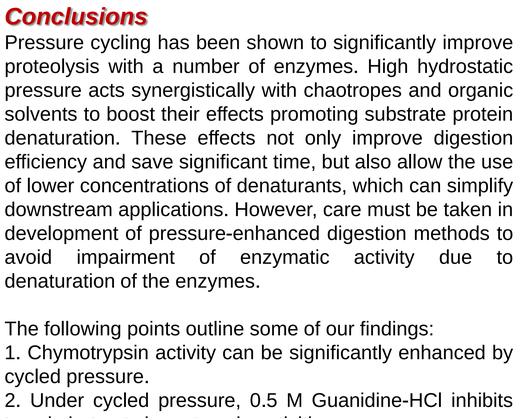

Figure 6: SDS PAGE of the first three serial dilutions of the mixture of six proteins (Hem – hemoglobin, CarAn – carbonic anhydrase, LaDe - lactate dehydrogenase, Alde - aldehyde dehydrogenase, pyKin – pyruvate kinase, and GlOx - glucose oxidase). The estimated amount of each protein loaded on the gel ranged from 4 µg (for the highest concentration, i.e. C_0) to 125 ng (for the lowest concentration, i.e., $C_0/32$). Each sample was loaded in triplicate.

Protein	Number of peaks detected, *S/N > 20		Number of peptides assigned to protein		% Sequence coverage	
	Conventional (±SD)	PCT (±SD)	Conventional (±SD)	PCT (±SD)	Conventional (±SD)	PCT (±SD)
Glucose Oxidase	97 (±21)	92 (±9)	14 (±2)	17 (±2)	38 (±4)	49 (±4)
Pyruvate Kinase	112 (±3)	128 (±17)	25 (±2)	30 (±2)	59 (±4)	66 (±1)
Catalase	86 (±2)	80 (±9)	18 (±2)	21 (±2)	45 (±5)	50 (±3)

Table 2: Summary of the comparison between the conventional and PCT-enhanced trypsin digestion in terms of number of peaks detected, number of peptides assigned to a protein and percentage of sequence coverage. Peptide identification was performed considering one missed cleavage. *S/N – signal-to-noise ratio.

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 their effects promoting substrate protein denaturation. These effects not only improve digestion efficiency and save significant time, but also allow the use of lower concentrations of denaturants, which can simplify downstream applications. However, care must be taken in development of pressure-enhanced digestion methods to avoid impairment of enzymatic activity due to denaturation of the enzymes.

The following points outline some of our findings:
1. Chymotrypsin activity can be significantly enhanced by 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 140 MPa exhibit negative effect on trypsin but not chymotrypsin activity (data not shown).
5. Under cycled pressure, trifluoroethanol below 15% enhances trypsin but inhibits chymotrypsin activity
6. Under cycled pressure urea can be included in trypsin digests concentration up to 1M, but become rapidly inhibitory at higher concentrations.

References

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