More Efficient Tissue Lysis and Protein Digestion with Lower Concentration of Denaturant Using Pressure Cycling Technology

Institute of Molecular Systems Biology IMSB

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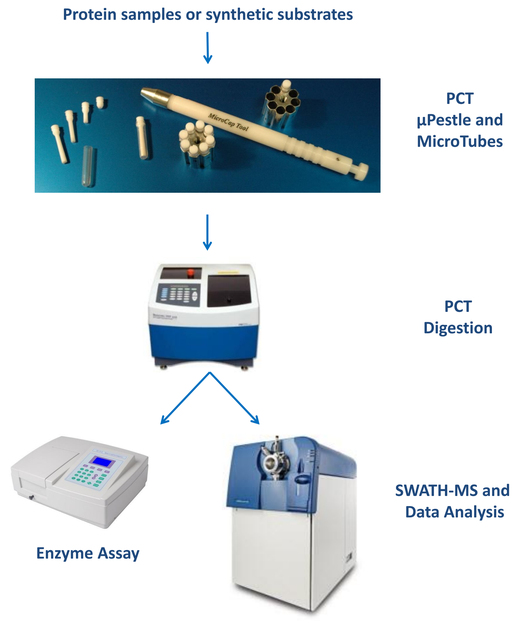
Abstract

In proteomic research, tissue lysis and protein digestion often require relatively high concentrations of denaturants to efficiently solubilize and denature proteins. Conventionally, 8 M urea is used to solubilize proteins, followed by digestion using Lys-C in 6 M urea and trypsin in 1.6 M urea. Such concentrations of urea may negatively influence the activity of proteolytic enzymes. Pressure cycling technology (PCT) is a unique methodology that offers programmed cycles oscillating between ultrahigh (up to 45,000 psi) and ambient (14.7 psi) pressure, which leads to efficient protein denaturation by hydrostatic pressure [1 - 3]. Moreover, pressure act in synergy with chaotropes, offering a potential to lower the amount of chemicals and streamline sample preparation steps. We have recently developed an integrated proteomic sample preparation protocol based on PCT that minimizes sample loss, and performs tissue lysis and accelerates digestion with a high degree of reproducibility [4]. We hypothesize that PCT could be employed to reduce the concentration of denaturants used in tissue lysis, leading to lower levels of denaturants in the digestion reactions, and boosting digestion efficiency by protecting the activity of proteolytic enzymes.

In this study, we tested the efficiency of PCT-based tissue lysis and protein digestion using different concentrations of urea buffer, and compared their performance based on peptide yield. We demonstrate that lysis by pressure cycling with the PCT µPestle in 4M urea substantially increased peptide yield compared to extraction in 8M urea. We further optimized urea concentration in the pressure-accelerated digestion reactions. To investigate the digestion kinetics under pressure, we utilized chromogenic substrates to measure enzyme activity during pressure cycling. We demonstrate that final urea concentrations about 0.8 M are compatible with high trypsin activity when cycling pressure at 20,000psi. Finally, we analyzed the peptides prepared from samples extracted with high concentration of urea and optimized lower concentration of urea, using the PCT-SWATH workflow.

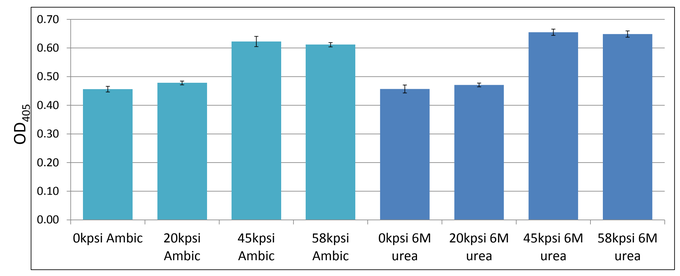
The efficiency of PCT-based tissue lysis and digestion is substantially improved by this optimized workflow with lower concentration of urea.

Pressure-Enhanced Protein Digestion Workflow

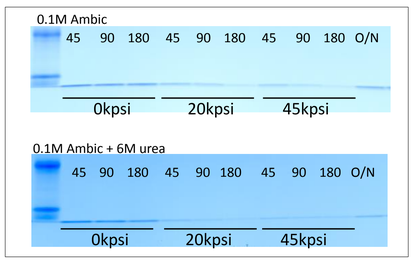


Optimization of Digestion with Lys-C

Lys-C activity assay with synthetic substrate +/- 6M urea at 20kpsi, 45kpsi and 58kpsi.
Lys-c activity is not negatively impacted by high concentrations of urea. Activity at 45kpsi is higher than at 20kpsi. Further increasing pressure to 58kpsi has no additional benefit, but does not appear to be detrimental. Results shown are average of triplicate samples ± standard deviation PCT conditions: 30 cycles, 50/10 at 35˚C in MicroTubes.



Lys-C digestion of a model protein.
Time course of ubiquitin digestion with Lys-C (45, 90, 180 mins) at 35˚C ± 6M urea. Overnight controls incubated at 35˚C in MicroTubes for ~20 hours. Samples were analyzed by SDS-PAGE. Loss of the intact parent protein indicates protein digestion by the enzyme.



Materials and Methods

Enzymatic Assays with Chromogenic Substrates

Chromogenic substrates BAPNA and N-p-tosyl-Gly-Pro-Lys 4-nitroanilide acetate were used to determine optimal pressure and urea concentration for maximum activity of trypsin (from Promega, sequencing grade) and Lys-C (from Wako). Enzyme activity was expressed as the slope of OD_{405} increase between 10 and 30 minutes of incubation of each enzyme with its chromogenic substrate.

Pressure-Enhanced Digestion of Model Protein

Digestion was performed at 35˚C with pressure cycling using the Barocycler NEP 3229 (Pressure BioSciences) and buffer conditions shown. Trypsin was used at enzyme-to-substrate (E:S) ratios of 1:260, and Lys-C at 1:40. 50ul aliquots of the reaction mixture were loaded into PCT MicroTubes. PCT conditions: 50 seconds at 20,000 or 45,000psi, 10 seconds at atmospheric pressure ("50/10"), per cycle. Control samples were incubated in PCT MicroTubes at 35˚C without pressure. Reactions were stopped by the addition of an equal volume of 2X Laemmli sample buffer supplemented with 0.5% formic acid.

Peptide Yield Measured by Nanodrop

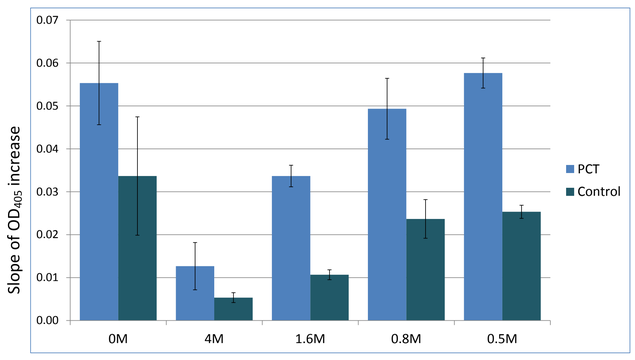
Digestion of 2-3mg mouse liver was performed with pressure cycling using Barocycler NEP2320-Enhanced in lysis buffer containing different urea concentrations (0-8M), 0.1M ammonium bicarbonate, COMPLETE protease inhibitor cocktail (Roche), and PhosSTOP phosphatase inhibitor cocktail (Roche). Pressure-accelerated digestion was performed with Lys-C (Wako, enzyme-to-substrate ratio 1:40) and Trypsin (Promega, enzyme-to-substrate ratio of 1:20) after tissue homogenization and extraction using the PCT µPestle with pressure cycling as published [4]. (Briefly, the extraction was performed for 60 cycles at 45,000psi. The Lys-C digestion was at 20,000psi for 45 cycles. The Trypsin digestion was at 20,000psi for 90 cycles. All at 35˚C). Subsequently, trifluoroacetic acid was added to the solution at a final concentration of 0.4% to stop digestion. The peptides were cleaned using SEP-PAK C18 cartridges (Waters Corp., Milford, MA), dried under vacuum, and resolved in HPLC grade water containing 0.1% formic acid and 2% acetonitrile. Peptide concentration was measured by absorbance at 280 using NanoDrop 1000 spectrophotometer.

Conclusions

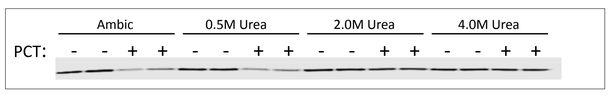
This study confirms earlier data suggesting that hydrostatic pressure can be used to accelerate enzyme digestions with trypsin and Lys-C. Pressure and urea concentration are being optimized to allow the entire extraction and digestion workflow to be carried out in a single 150 uL MicroTube sample container. Lys-C appears to be much more stable at high pressure and high urea concentrations, compared to trypsin. Moreover, Lys-C activity is significantly enhanced at high pressure, enabling more efficient and rapid digestion immediately following protein extraction in urea-containing buffers. Subsequent tryptic digestion is also improved by further dilution of urea to 0.8M final concentration. Thus, the entire workflow could be carried out in a single MicroTube by preforming extraction in 30uL of 4M urea followed by Lys-C digestion and dilution to 0.8M urea in 150 uL for Trypsin digestion. Moreover, pressure-enhanced digestion produces a greater number of proteotypic peptides even when short digestion protocols are employed. Thus, pressure can be used to generate more reproducible digests using shorter digestion times, leading to higher throughput.

Optimization of Digestion with Trypsin

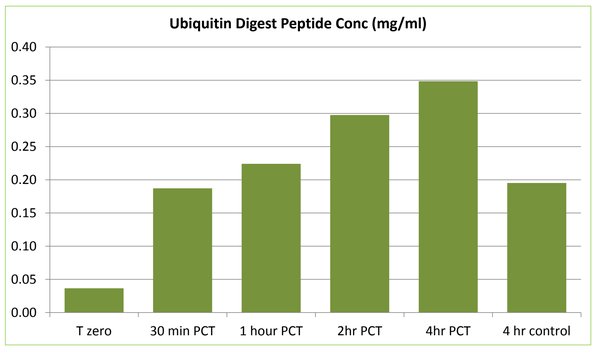
Trypsin activity assay (slope calculated from OD_{405} at 10 and 30mins) with synthetic substrate +/- different concentrations of urea at 20,000 psi. Total time at 35˚C was the same for all samples. Results shown are average of triplicate samples ± standard deviation.



Pressure-accelerated trypsin digestion of a model protein (bovine ubiquitin) with different concentrations of urea, demonstrating the need to optimize urea concentration (+PCT = digestion with pressure cycling at 20,000psi , -PCT = digestion at ambient pressure).



Trypsin digestion of ubiquitin +/- PCT. Time course (in 50mM ammonium bicarbonate, no urea) and peptide quantification. Peptides separated from parent protein using 3k MWCO filter and quantified by BCA (duplicate samples). PCT 20kpsi, 50˚C, 30-240 cycles (50/10) in PCT MicroTubes.

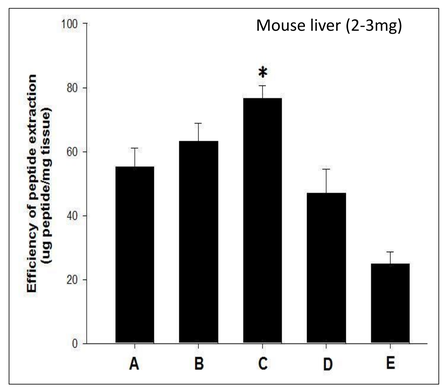


Optimization of Urea Concentration in Sequential Lys-C/Trypsin Protocol

The efficiency of peptide yield is highest in samples extracted in 4M urea, followed by digestion with Lys-C in 3M urea and then with trypsin in 0.8 M urea. We demonstrate that homogenization and extraction by pressure cycling with the PCT µPestle in 4M urea followed by the optimized digestion conditions, substantially increased peptide yield over samples processed in higher urea concentrations.

Comparison between samples extracted with PCT µPestle in different urea concentration.

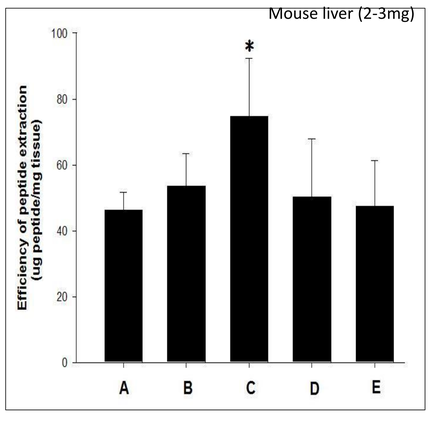
Peptide concentration measured by Nanodrop. Yield expressed as ug peptide per mg of tissue. Data presented are the mean ± SEM of three independent experiments.
A. 8M urea in lysis buffer, followed by digestion using Lys-C in 6M urea and trypsin in 1.6M urea. **B**. 6M urea in lysis buffer, followed by Lys-C in 4.5M urea and trypsin in 1.2 M urea. **C**. 4M urea in lysis buffer, followed by Lys-C in 3M urea and trypsin in 0.8 M urea. **D**. 2M urea in lysis buffer, followed by Lys-C in 1.5M urea and trypsin in 0.4M urea. **E**. No urea in lysis or digestion buffers.



Based on the above result, we then further optimized the pressure-accelerated digestion conditions, using samples extracted with PCT µPestle in 4M urea. The best peptide yield was found in samples digested at high pressure with Lys-C in 4M urea, followed by trypsin digestion at high pressure in 0.8M urea.

Comparison between samples extracted with PCT microPestle using different urea concentration.

Peptide concentration measured by Nanodrop. Yield expressed as ug peptide per mg of tissue. Data presented are the mean ± SEM of three independent experiments.
A. 8M urea in lysis buffer, followed by Lys-C in 6M urea and trypsin in 1.6M urea. **B**. 4M urea in lysis buffer, followed by Lys-C in 4M urea and trypsin in 0.8M urea. **C**. 4M urea in lysis buffer, followed by Lys-C in 3M urea and trypsin in 0.8M urea. **D**. 4M urea in lysis buffer, followed by Lys-C in 2M urea and trypsin in 0.8M urea. **E**. 4M urea in lysis buffer, followed by Lys-C in 1M urea and trypsin in 0.8 M urea.



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