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

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Abstract

In proteomic research, tissue lysis and protein digestion often require relatively high concentrations of denaturants to efficiently solubilize proteins and denature proteins. Conventionally, 8 M urea is used to solubilize proteins, followed by digestion using LysC 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 pHiFi in 4 M urea substantially increased peptide yield compared to extraction in 8 M urea. We further optimized urea concentration in the pressure-accelerated digestion reactions. To investigate the 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, and we utilized the peptide recovered from samples extracted with high concentration of urea and optimized lower concentration of urea, using the PCT-pHiFi workflow. The efficiency of PCT-based tissue lysis and digestion is substantially improved by this optimized workflow with lower concentration of urea.

Materials and Methods

Enzymatic Assays with Chromogenic Substrates

Chromogenic substrates BAPA 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 LysC (from Wako). Enzyme activity was expressed as the slope of OD405. Following between 10 and 30 minutes of incubation of each enzyme with its respective 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 1:1.26, and Lys-C at 1:40. 50 ul 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 2-8 mg mouse liver was performed with pressure cycling using Barocycler NEP3220. Enzymes in lysate buffer containing different urea concentrations (0-8M), 0.1M ammonium bicarbonate, COMPLETE protease inhibitor cocktail (Roche), and PhosSTOP phosphatase inhibitor cocktail (Roche) were pressure-accelerated digestion performed with LysC (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 pHiFi with pressure cycling as published [4]. Briefly, the extraction was performed for 60 cycles at 45,000 psi. The LysC digestion was at 20,000psi for 45 cycles. The Trypsin digestion was at 20,000psi for 90 cycles. All at 35°C. Subsequently, 2,4-trifluoracetic acid was added to the solution at a final concentration of 0.4% to stop digestion. The peptides were cleaned using SE-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.

Optimization of Digestion with Trypsin

Trypsin activity assay (slope calculated from OD405 at 10 and 30min) with synthetic substrate (+/- different concentrations of urea at 20,000 psi. Total time at 35°C was the same for all samples. Results show are average of trypsin samples ± standard deviation.

Conclusions

This study confirms earlier data suggesting that hydrostatic pressure can be used to accelerate enzyme digestions with trypsin and LysC. 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. LysC appears to be 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 preextracting in 30ul of 4M urea followed by LysC 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.

References


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