PCT-HD: Homogenization, Extraction and Digestion of Small Tissue Samples for Proteomic Applications.

Introduction

The PCT-HD system is a combination of two of PBI's innovative sample preparation tools; the NEP2320-Enhanced Barocycler and PCT MicroPestles [1]. This unit can generate a maximum pressure of 45 kpsi (the original NEP2320 Barocycler is rated to 35 kpsi) and can be heated to temperatures up to 95^0 C. The higher pressure and temperature range give the NEP2320-Enhanced broad applicability in proteomics, enzymology and other fields of research.

Pressure Cycling Technology (PCT) has been proven to accelerate enzymatic protein digestion. The positive effect of PCT on trypsin digestion is well established [2, 3, and 4] for improved sequence coverage, higher recovery and significantly reduced digestion times. Not only has PCT been shown to accelerate and improve digestion in solution, but it can also accelerate in-gel trypsin digestion [5, 6]. Additionally, the enhancing effect of PCT on the activity of several other enzymes, including Proteinase K, PNGase F, chymotrypsin and lysozyme has been reported [7, 8, and 9].

The PCT-HD workflow starts with small solid tissues, such as biopsies, and ends with peptide samples ready for clean-up and MS, or other analyses. The entire procedure, from tissue to peptides, can be performed in ~4 hours, with up to 16 samples processed simultaneously [10, 11]. Depending on the sample type and the downstream clean-up required, the procedure may be carried out in a single MicroTube without any tube-to-tube transfers, thus significantly reducing sample loss and cross-contamination between samples.

After sample homogenization/extraction with the PCT MicroPestle, pressure-accelerated digestion is carried out using Lys-C followed by Trypsin. High hydrostatic pressure accelerates protein digestion via a combination of two mechanisms. The first is the pressure-induced partial denaturation/unfolding and hydration of the target proteins in the sample, which leads to better access of the enzyme to its target sites. The second mechanism is less well understood, but relies on the increase in enzyme activity observed for some enzymes at elevated pressure. This increase in activity can be demonstrated when enzyme assays are carried out at high pressure using small peptide substrates that are not expected to exhibit significant unfolding or hydration at high pressure, due to their small size. The combination of the two pressure-based mechanisms, one acting on the enzyme and the other acting on the substrates, leads to significantly accelerated digestion, but may need to be optimized for each enzyme to prevent pressure-induced denaturation and loss of activity of the enzyme itself. For this reason, the digestions with Lys-C and trypsin are carried out sequentially, at different pressures and with slightly different buffer compositions. This sequential digestion allows each reaction to occur under optimal pressure and buffer conditions.

PCT-HD Workflow and Optimization.

Protein Extraction by PCT with µPestles

Homogenization and extraction from small tissue samples (<3.0 mg) requires protocols that minimize sample dilution and loss. PCT µPestles (**Figure 1**) are designed for processing up to 3 mg of soft solid tissue in 30 µl of extraction reagent. Once inserted into the MicroTubes, the tight-fitting µPestles force the tissue into the small space available. During pressure cycling, the flexible tube is repeatedly forced against the rigid µPestle, resulting in efficient tissue homogenization and cell lysis. To maximize protein recovery, the µPestles are made of PTFE (aka Teflon® by DuPont) and the MicroTubes are made of FEP, an inert, non-wetting Teflon-like material that has very low protein binding properties.

Figure 1

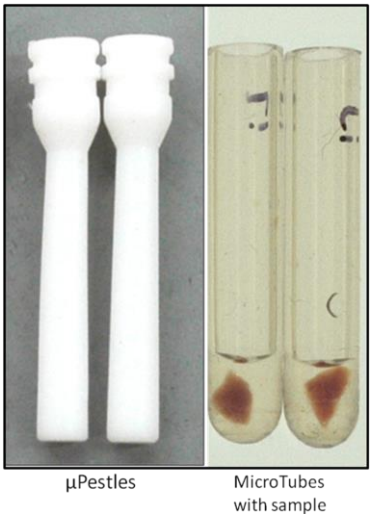


Figure 1. Appearance of PCT µPestles and MicroTubes. MicroTubes are shown with ~3mg of liver tissue and 30 µl of extraction reagent.

Tissue Homogenization by PCT in a PBI Barocycler with µPestles allows for significantly reduced hands-on time, increased throughput, and minimal sample loss, compared to standard manual homogenization methods. Initial optimization of sample extraction using PCT µPestles was carried out previously [12] using the standard NEP2320 Barocycler at pressures up to 35 kpsi. Those studies showed that up to 3 mg of tissue could be processed, although best results were obtained when sample mass was kept below 2 mg. The optimal number of pressure cycles for extraction was found to be 60, although for softer samples, such as liver, as few as 20 cycles were generally sufficient. Since the 2320-Enhanced Barocycler can reach a pressure of 45 kpsi, additional tests were carried out to determine whether protein yield could be improved by performing the tissue homogenization at 45 kpsi. Results of this testing (**Figure 2**) suggest that for relatively soft tissues, such as liver, increasing pressure from 35 to 45 kpsi results in little improvement in protein yield; however, for tougher tissues like muscle, increasing the pressure to 45 kpsi leads to a significant improvement in total protein yield.

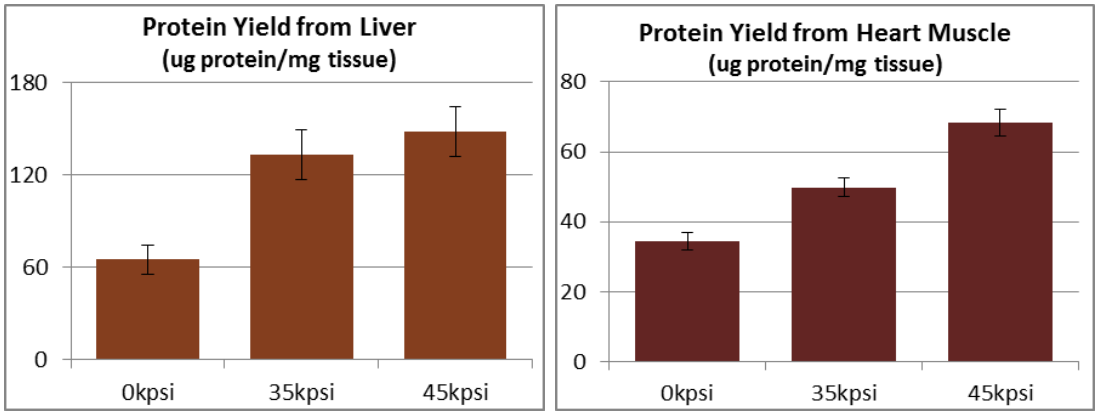


Figure 2. Effect of pressure level on MicroPestle tissue homogenization and total protein yield. Samples homogenized using the PCT MicroPestle for 30 cycles at the indicated pressure (liver tissue extraction n=10, avg ±SEM, heart muscle tissue extraction n=20, avg ±SEM) in lysis buffer containing 8M urea and 100mM ambic. Protein concentration measured by BCA assay.

The PCT µPestle method is an effective tool for rapid and convenient disruption and extraction of small solid tissue samples, such as liver, brain, lung, etc. Under high hydrostatic pressure, the air in the MicroTube becomes compressed, causing the MicroTube to flex significantly. As a result, the tissue sample trapped between the MicroTube and the µPestle tip is crushed on each pressure cycle. This mechanical action, combined with high pressure sample lysis, results in effective homogenization and extraction.

PCT-Enhanced Lys-C Digestion

PCT has been shown to accelerate and improve digestion of proteins by several enzymes, including the proteolytic enzyme Lys-C [9]. To optimize pressure-enhanced Lys-C digestion, colorimetric activity assays as well as model protein digests were used. Myoglobin is a small, soluble protein that is poorly digested by Lys-C under normal conditions, and therefore is a good model of a “difficult-to-digest” protein. Optimization of pressure-accelerated Lys-C digestion for the PCT-HD workflow demonstrated that best

results were obtained at 45,000psi in 3M urea at 35^0 C (**Figure 3**). Further optimization of Lys-C digestion is described in detail in a separate Application Note [9].

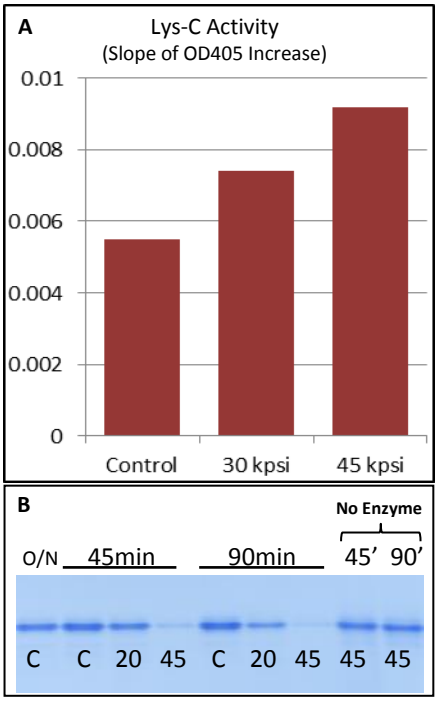
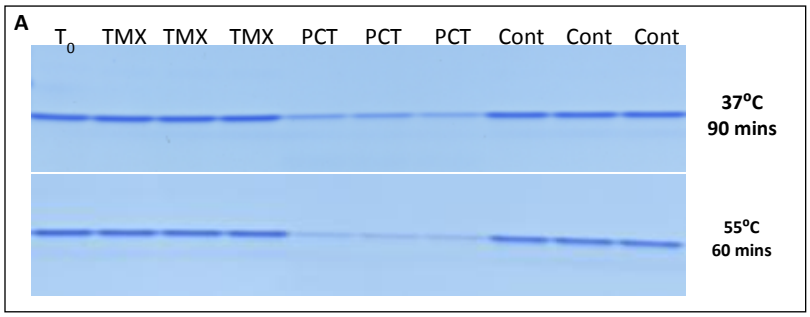
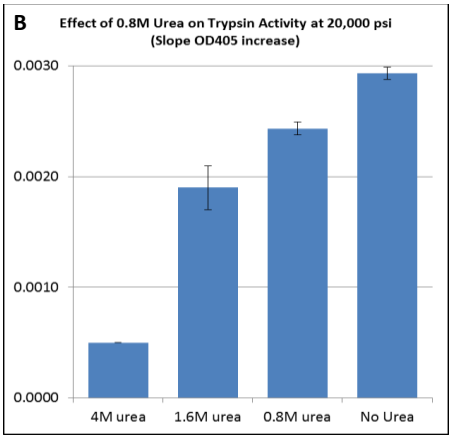


Figure 3. Effect of pressure cycling on Lys-C activity. **A**. Enzyme activity assay with synthetic substrate shows the increase in Lys-C activity at high pressure (increasing slope = increasing rate of reaction). **B**. Digestion of Myoglobin with Lys-C in 3M urea at 35^0 C. Reactions carried out at ambient pressure (C), 20kpsi or 45kpsi for 45 or 90 minutes. Negative controls were incubated at 45 kpsi without enzyme for 45 or 90 mins. An overnight digest control (O/N) demonstrates poor digestion of myoglobin by Lys-C under normal conditions. The model protein digests agree with the enzyme activity assay and confirm that Lys-C is significantly accelerated by pressure cycling at 45 kpsi.

PCT-Enhanced Trypsin Digestion

PCT-enhanced trypsin activity was measured using both a colorimetric activity assay, and a model protein as the substrate. Ubiquitin, which like myoglobin is a small, soluble protein, is poorly digested by trypsin under normal conditions, making it a good model of a "difficult-to-digest" protein. **Figure 4A** shows the acceleration of ubiquitin digestion by trypsin under PCTconditions at 20 kpsi. Previous optimizations [9] demonstrated that high concentrations of urea were detrimental to trypsin activity under pressure. Here we expand upon that finding and demonstrate that at low concentration (up to 0.8M), the effect of urea on trypsin activity at 20 kpsi is relatively slight and is compatible with the PCT-HD workflow (**Figure 4B**).





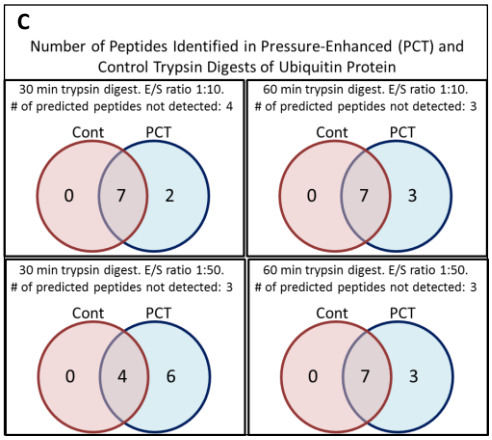


Figure 4. Effect of high pressure cycling at 20 kpsi on trypsin activity. **A**. Trypsin digestion of native ubiquitin protein in 100 mM ammonium bicarbonate. Samples were incubated at either 37^0 C for 90 mins or 55^0 C for 60 mins. Control samples incubated in waterbath without shaking or in thermomixer (TMX) with shaking (n=3). **B**. Trypsin activity assay with synthetic substrate at 20 kpsi with different concentrations of urea. These data show that there is a slight reduction in trypsin activity when the reaction mixture is supplemented with 0.8M urea, but that the inhibition is much greater at higher concentrations of urea (n=3 ± std dev). **C.** Comparison of the number of peptides detected in trypsin digests of ubiquitin processed with or without PCT. Reactions were carried out in 50mM ambic supplemented with 10% N-propanol for 30 or 60 minutes, with or without PCT at 20 kpsi using the indicated enzyme-to-substrate (E/S) ratio. The total number of predicted tryptic peptides for this protein is 13.

PCT-Enhanced Sequential Digestion by Lys-C and Trypsin

A mixture of myoglobin and ubiquitin was used to test the conditions for sequential digestion with Lys-C and trypsin. As shown in **Figure 5**, PCT significantly improves the digestion of these difficult-to-digest model proteins, notably ubiquitin, which is almost entirely intact in the control digests performed without pressure. Note that the Lys-C digest shown in Figure 5 was carried out at 20kpsi, which has been shown to accelerate Lys-C to a lesser extent than at 45 kpsi (see **Figure 4A**). When carried out at 45kpsi the Lys-C reaction proceeded so quickly, that both protein bands were almost completely absent from the gel (data not shown) making it impossible to visualize the sequential digestion by trypsin.

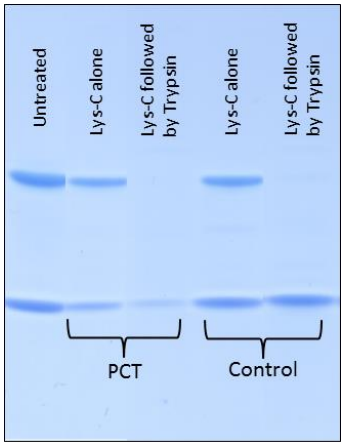


Figure 5. Sequential pressure-accelerated digestion of model proteins with Lys-C followed by Trypsin demonstrates improved digestion of difficult-to-digest proteins.
A protein solution containing a 1:1 mixture of myoglobin and ubiquitin was digested with either Lys-C alone or Lys-C followed by trypsin. 30µl of protein stock in 4M urea/100mM ambic was placed into each MicroTube. The protein sample was diluted to 40µl (to 3M urea) for digestion by lys-C (E/S 1:75) with or without PCT at 20 kpsi for 30 mins. The sample was supplemented with n-propanol and further diluted to 145µl (to 0.8M urea, 15% n-propanol) prior to the addition of trypsin (E/S 1:60). Trypsin digestion was carried out for 90 mins, with or without PCT at 25kpsi. All samples were adjusted to 145µl final volume prior to addition of 2x SDS-PAGE loading buffer.

Discussion

Treatment with high hydrostatic pressure has been shown to accelerate digestion by trypsin, Lys-C, [7, 8, and 9] and other enzymes. The increase in apparent enzyme activity can occur via one or both of the two mechanisms described above. The effect of PCT on trypsin activity appears to be due primarily to pressure-induced denaturation of the substrate protein rather than by pressure-effects on the activity of the enzyme itself. By forcing substrate proteins into an unfolded/hydrated state, PCT effectively makes them “better” substrates for the enzyme. For this reason, it is recommended that trypsin digestion be carried out at the lowest pressure at which effective substrate protein denaturation can be achieved. We suggest pressure around 20 kpsi, since denaturation and reduction of trypsin activity may occur at higher pressure levels. However, for certain “very-hard-to-unfold” proteins, it may be possible to obtain better digestion results by performing pressure-accelerated trypsin digestion at slightly, or even significantly, higher pressure (e.g., 25-45 kpsi).

The activity of Lys-C, on the other hand, appears to be accelerated by PCT via a combination of the two mechanisms. Colorimetric activity assays suggest that Lys-C activity is accelerated at high pressure independent of the effect of pressure on the substrate; although it is likely that pressure-induced denaturation of the substrate protein also contributes to the improved digestion of the model protein by Lys-C under pressure.

Based on the results described here and reported elsewhere [4, 10, 11, 13, and 14], a suggested workflow for the PCT-HD method is shown below. Using the guidelines in Tables 1-3, this protocol can be used “as-is”, or as a starting point for further optimization, to design pressure-enhanced protocols for specific needs and sample types.

Suggested workflow for PCT-HD:

1. Place tissue sample (<3 mg) into PCT MicroTube.
2. Add 30 µl extraction reagent (4M urea, 100mM ammonium bicarbonate).
3. Cap with PCT µPestle.
4. Place into NEP 2320-Enhanced Barocycler.
5. Perform pressure cycling at 45 kpsi for 60 cycles (20 sec at high pressure, 10 sec at ambient pressure, per cycle).
6. Remove tubes from Barocycler.
7. Withdraw and discard µPestles.
8. Place MicroTubes into small centrifuge tubes and centrifuge briefly to bring sample down to the bottom of the MicroTubes.
9. Reduce/Alkylate the samples using standard methods. Cap tubes with "50 µl" MicroCaps.
10. Add Lys-C enzyme and 100mM ammonium bicarbonate to bring the total volume to ~40 µl (this brings the urea concentration to 3M). Enzyme-to-substrate ratio should be ~1:50, but can be adjusted up/down to optimize digestion of different samples. Re-cap with same "50 µl" MicroCaps.
11. Perform pressure-accelerated digestion (PCT) at 35^0 C, using 20-45kpsi for 30-60 cycles (50 sec at high pressure, 10 sec at ambient pressure, per cycle).
12. Withdraw and discard used MicroCaps. Tubes can be centrifuged briefly, if desired.
13. Add Trypsin enzyme and 100mM ammonium bicarbonate to bring the total volume to ~145 µl (this brings the urea to 0.8M). Enzyme-to-substrate ratio should be ~1:20 to 1:50, but can be adjusted up/down to optimize digestion of different samples. Cap with "150 µl" MicroCaps.
    Note: Addition of 10-15% N-propanol (final concentration) is optional, but may improve digestion efficiency.
14. Perform pressure-accelerated digestion at 35^0 C, using 20 kpsi for 60-90 cycles (50 sec at high pressure, 10 sec at ambient pressure, per cycle).
15. Proceed to sample clean-up using standard methods.

Table 1. Suggested Starting Parameters for Optimization of the PCT-HD protocol

Step	Parameter	Suggested Starting Condition
Tissue Homogenization and Extraction	Sample container	µPestle and MicroTube
	Pressure cycling conditions	45 kpsi, 20 seconds at time 1, 10 seconds at time 2, 20-60 cycles depending on softness of the tissue
	Temperature	Ambient
	Buffer	Ammonium bicarbonate with 4M urea
	Volume	≤30 µl per MicroTube (do not use more than 30 µl)
Digestion with Lys-C	Sample container	MicroTubes with "50ul" MicroCaps
	Pressure cycling conditions	20-45 kpsi, 50 seconds at time 1, 10 seconds at time 2, 30-60 cycles.
	Temperature	35°C (or 50-55^0 C, if **not** using urea)
	Buffer	Ammonium bicarbonate, 3M urea
	Denaturant (optional)	10% n-propanol
	Volume	~40 µl per MicroTube
Digestion with Trypsin	Sample container	MicroTubes with "150ul" MicroCaps
	Pressure cycling conditions	20 kpsi, 50 seconds at time 1, 10 seconds at time 2, 60-90 cycles.
	Temperature	35°C (or 50-55^0 C, if **not** using urea)
	Buffer	Ammonium bicarbonate, ≤0.8M urea
	Denaturant (optional)	10-15% n-propanol
	Volume	~150 µl per MicroTube. Note: this volume is based on the required dilution of urea in the lysis buffer. If no urea is present, the volume can be reduced to 50 or 100 µl. Be sure to use the appropriate length MicroCap.

Table 2. Compatibility of Denaturants with Pressure-Enhanced Lys-C Digestion

Denaturant	Compatibility	Notes
Urea	Optimal at 3M. Can be used at 0-6M.	Higher concentrations may slow reaction. If urea is omitted, temperature should be increased to 50-55^{0}C to compensate.
Acetonitrile	Compatible at up to 20% final concentration.	At 40%, will inhibit reaction.
n-Propanol	Recommended at 10% final concentration.	Higher concentrations may inhibit reaction.

Table 3. Compatibility of Denaturants with Pressure-Enhanced Trypsin Digestion

Denaturant	Compatibility	Notes
Urea	Compatible at or below 0.8M final concentration.	Higher concentrations may slow or inhibit reaction.
Acetonitrile	Compatible at 10% final concentration.	Higher concentrations will inhibit reaction.
n-Propanol	Recommended at 10-15% final concentration.	Higher concentrations may inhibit reaction.

Materials and Methods

Lys-C (mass spectrometry grade) was purchased from Wako. Trypsin (sequencing grade) was purchased from Promega. Equine myoglobin, ubiquitin, chromogenic substrates N-p-Tosyl-Gly-Pro-Lys-pNA (for Lys-C assays) and N_α-Benzoyl-D,L-argenine 4-nitroanilide hydrochloride (for trypsin assays) were purchased from Sigma. PCT was performed either in an NEP2320-Enhanced Barocycler or in an SW16 pressure chamber attached to a HUB 440 Barocycler. Both units were equipped with built-in temperature control. Reactions were carried out in 100mM ammonium bicarbonate (ambic) unless indicated otherwise.

Lys-C activity was measured at 35^0 C using a chromogenic substrate in 100 mM ambic supplemented with 3M urea. Trypsin activity was measured using the chromogenic substrate, N_α-Benzoyl-D,L-argenine 4-nitroanilide hydrochloride (BAPNA). BAPNA assays were carried out in 100 mM ambic supplemented with urea at the indicated concentration, using 2 µg/ml trypsin and 500 µg/ml BAPNA. All reactions were carried out in triplicate using 150 µl of reaction per MicroTube. PCT was performed in PCT MicroTubes using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure per cycle). Control reactions were incubated in MicroTubes at the same temperature but without pressure. OD405 was measured at multiple (2 or more) time points and used to calculate the slope of the OD405 increase.

Model protein digestion by Lys-C and/or trypsin was assayed using native myoglobin and native ubiquitin as substrates. PCT was performed in PCT MicroTubes, using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure per cycle) at the indicated pressure. Control reactions were incubated in MicroTubes at the same temperature but without pressure. Enzyme-to-substrate ratio was 1:50-1:100 unless indicated otherwise. SDS-PAGE was carried out using Criterion gels (Bio-Rad). Proteins were visualized by staining with coomasie blue (Bloo Moose Staining Solution, Kerafast). Protein digestion was assayed by SDS-PAGE as the disappearance of the intact parent protein band.

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