Compatibility of Deoxycholate Detergent with Pressure-enhanced Digestion by Trypsin and Lys-C: Alternative PCT-HD Workflow

Introduction

The PCT-HD workflow [1] is a combination of two of PBI's innovative sample preparation tools; the 2320EXT Barocycler and PCT MicroPestles [2, 3]. Together they utilize pressure cycling technology (PCT) to extract and digest proteins from biopsy-size tissue samples. PCT has been proven to accelerate enzymatic protein digestion; the positive effect of PCT on digestion by both trypsin and Lys-C is well established [4-7]. Additionally, the enhancing effects of PCT on the activity of enzymes including Proteinase K, PNGase F, chymotrypsin and lysozyme have been reported [8-11].

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 or less, with up to 16 samples processed simultaneously. 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 the risk of cross-contamination between samples.

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, 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, possibly resulting from the effect of pressure on hydrolysis. 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 and reagent system, to prevent pressure-induced denaturation and loss of activity of the enzyme itself. To this end, the effect of sodium deoxycholate (DOX) on PCT-accelerated trypsin and lys-C digestions, has been evaluated.

Effect of DOX on MicroPestle extraction and PCT-enhanced digestion of model proteins

Homogenization/extraction in the PCT-HD workflow is usually carried out in lysis buffer containing 4M urea. The urea is then sequentially diluted, first to 3M for lys-C digestion, and then to 0.8M for trypsin. One significant drawback of urea-based lysis buffers is the urea-induced carbamylation of peptides that occurs at elevated temperatures. For this reason, when urea-containing buffers are used, proteomic samples must be kept at or below 37C throughout the sample preparation process. In buffers without urea, PCT-accelerated digestion can be carried out at 50-55C, allowing for a much shorter sample processing time. In addition, previous optimizations [5] suggested that even moderate amounts of urea (~2M), could be detrimental to trypsin activity under pressure. Here we examined the effect of DOX as a substitute for urea in the PCT-HD workflow.

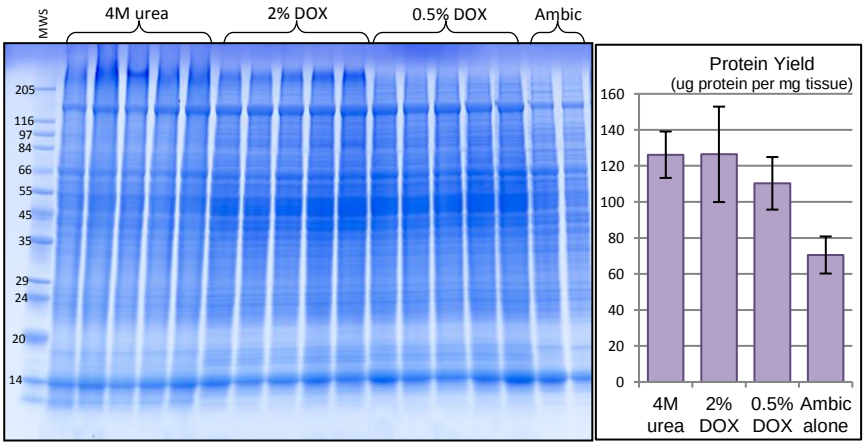


Figure 1. Tissue protein extraction with PCT MicroPestle; effect of lysis buffer composition. Protein profile assayed by SDS-PAGE. Yield quantified by BCA assay. Protein profile of lysates on SDS-PAGE suggests that different populations of protein are recovered in 4M urea compared to 2% DOX. Overall, 2% DOX appears to result in more bands. Reducing the DOX to 0.5% results in slightly lower yield and loss of the very high molecular weight proteins (above the 205 kDa molecular weight standard band). Total protein yield in 2% DOX is comparable to that obtained in 4M urea. Reducing the DOX concentration to 0.5% reduces the yield by only ~15%. As expected, protein yield is significantly lower when the samples are prepared in ammonium bicarb alone. Values shown are averages of 5-10 replicates. Error bars indicate Std Dev.

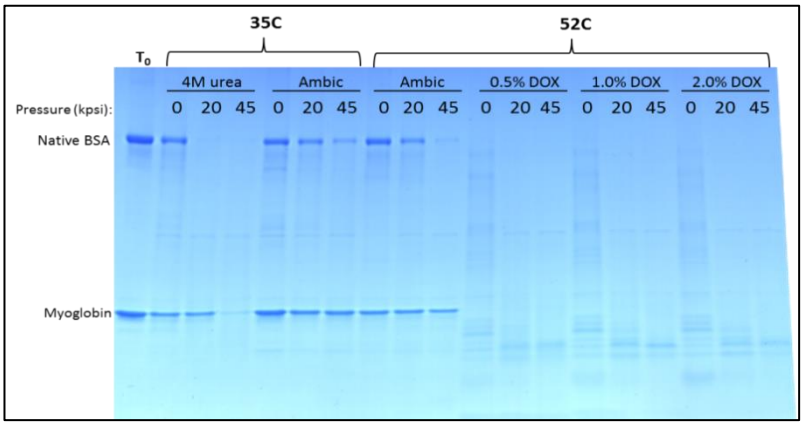
We compared total protein yield from rat liver tissue extracted using the PCT MicroPestle, in 4M urea, 2% DOX, and 0.5% DOX. Control extractions were carried out in ammonium bicarb with no chaotropes or detergents. The results (Figure 1) clearly show that lysis buffer made with 2% DOX recovers as much protein as 4M urea. Even 0.5% DOX in the lysis buffer can be used, with only a slight reduction in overall protein yield. In addition, tissue lysis in 2% DOX appears to increase the number of protein bands seen on SDS-PAGE, suggesting that extraction in DOX may result in increased proteomic coverage.

Effect of DOX on PCT-enhanced Lys-C digestion

Difficult-to-digest model proteins, known to be inefficiently digested by both Lys-C and trypsin under normal conditions, were used to examine pressure-enhanced digestion in urea and DOX. Protein digestion was assayed by SDS-PAGE as the disappearance of the intact protein band [1, 5, 6]. For all reagents tested, control digests were carried out under the same conditions (time and temp), but at ambient pressure ("0 kpsi"). The results (Figure 2) confirm that Lys-C digestion proceeds more quickly in 4M urea than in buffer without chaotrope (ambic alone) at 35C. In the presence of DOX, at all three concentrations tested, the Lys-C digestion at 52C proceeds significantly faster than in urea at 35C. The combined effect of pressure and DOX can be seen in samples digested with pressure (both 20 and 45kpsi), which show significantly better digestion compared to controls incubated at ambient pressure.

PCT-Enhanced trypsin digestion in the presence of DOX

Figure 2. Effect of DOX on Lys-C digestion of model proteins at ambient pressure, and at 20 or 45 kpsi. Model proteins (native BSA and myoglobin) digested with Lys-C for 1 hour. Digests in ambic and in 0.5%, 1% and 2% DOX were carried out at 52C. For comparison, digests in ambic alone and in 4M urea were carried out at 35C. Digestion at elevated pressure (20 and 45kpsi) in the presence of DOX resulted in significantly better digestion of the model proteins, compared to digestion at ambient pressure (0kpsi).



To optimize pressure-enhanced trypsin digestion in the presence of DOX, model proteins were digested at 20kpsi and 45kpsi in the presence of varying concentrations of the detergent. Control digests were carried out under the same conditions (time and temp) at ambient pressure ("0 kpsi"). Additional controls were incubated at ambient pressure for 4 hours.

The results (Figure 3) demonstrate that pressure cycling in the presence of DOX is more effective than either DOX alone or pressure alone for digestion of myoglobin and disulfide-intact BSA. The results also show that ubiquitin is digested most effectively at 45kpsi, in the presence of little or no DOX. These results highlight the fact that different protein targets may require slightly different conditions for optimal digestion.

Based on the 3 model proteins used here, the best condition for trypsin digestion in DOX-containing buffer is at 45kpsi in ~0.05-0.4% DOX at 52^0 C. Interestingly, 0.05% DOX appears to have an overall slightly negative effect on BSA digestion by trypsin, compared to digestion either without any DOX, or digestion with higher concentrations of DOX (Figure 3). The reason for this observation is not clear, but may be due to a crossing-over point between 2 effects of DOX; a slight negative effect of DOX on trypsin activity, that can be counterbalanced by the positive effect of DOX on unfolding of the substrate protein. It is possible that at 0.05% DOX, there is not enough detergent to effectively unfold the BSA, but enough to slightly affect the structure and activity of the trypsin. This observation further highlights the fact that different protein targets may require slightly different conditions for optimal digestion.

Nevertheless, the results clearly demonstrate the benefit of PCT for accelerating trypsin digestion. Reactions carried out at ambient pressure, even after 4 hours, show significantly less protein digestion compared to reactions treated with pressure cycling for 90 minutes. Additional benefit is derived when the DOX-containing samples are digested at 52^0 C, a temperature that is not commonly used with urea-containing buffers as this would lead to very high rates of peptide carbamylation.

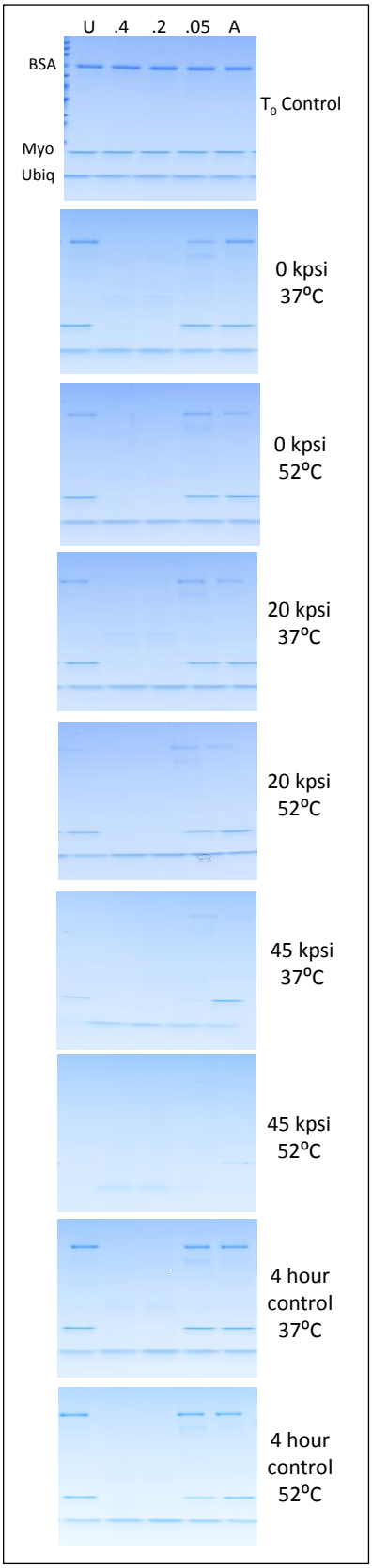


Figure 3. **Effect of temperature and DOX concentration on trypsin digestion of model proteins at atmospheric pressure ("0 kpsi"), 20kpsi and 45kpsi.** Model proteins (native BSA, ubiquitin, and myoglobin) digested with trypsin for 90 minutes at the indicated pressure and temperature. Undigested (T_0) proteins are shown for comparison (top panel). Control reactions (bottom 2 panels) were digested at atmospheric pressure for 4 hours at 37 or 52^0 C. Reactions were carried out in 100mM ambic (A), ambic supplemented with DOX at the indicated concentration (0.05%, 0.2% or 0.4%), or in ambic with 0.8M urea (U).

Discussion

Based on the results described here, the PCT-HD workflow can be adapted to use DOX, rather than urea, in the lysis buffer. Using the workflow described below, 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.

When designing PCT-enhanced digestion conditions, it should be kept in mind that by forcing substrate proteins into an unfolded/hydrated state, PCT effectively makes them "better" substrates for enzyme digestion. However, since hydrostatic pressure, and the presence of chaotropes and/or detergents, can also affect the conformation of the enzyme itself, it is generally recommended that digestion be carried out at the lowest pressure, and with the least amount of detergent, at which effective accelerated digestion can be achieved. For this reason, we suggest 20 kpsi as a starting condition for trypsin. However, for many "hard-to-unfold" proteins (such as ubiquitin), significantly better digestion results can be achieved by performing trypsin digestion at 45 kpsi. Conversely, Lys-C has been shown to be much more pressure-stable than trypsin. Colorimetric activity assays [6] 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. Therefore, for lys-C digestion, we recommend 45 kpsi as the starting pressure.

Suggested workflow for PCT-HD with deoxycholate:

1. Place tissue sample (<3 mg) into PCT MicroTube.
2. Add 30 µl extraction reagent containing ~1-2% DOX in 100mM ammonium bicarbonate (other buffers may also be used).
3. Cap with PCT µPestle.
4. Place into 2320EXT Barocycler and perform pressure cycling at 45 kpsi for 60 cycles (20 sec at high pressure, 10 sec at ambient pressure, per cycle).
5. Remove tubes from Barocycler and discard µPestles.
6. (Optional) Place MicroTubes into small centrifuge tubes and centrifuge briefly to bring sample down to the bottom of the MicroTubes.
7. Reduce/Alkylate the samples using standard methods. Use a long "50 µl" MicroCap for this step. The same cap can be re-used in the next step.

8. Add Lys-C enzyme and 100mM ammonium bicarbonate to bring the total volume to ~40 µl (bringing the DOX concentration to ~0.75-1.5%, depending on starting concentration). 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" MicroCap.
9. Perform Lys-C digestion in Barocycler at 52^0 C, using 45 kpsi for 30-60 cycles (50 sec at high pressure, 10 sec at ambient pressure, per cycle).
10. Withdraw and discard used MicroCaps. Tubes can be centrifuged briefly, if desired.
11. Add Trypsin enzyme and 100mM ammonium bicarbonate to bring the total volume to ~145µl (this brings the DOX concentration down to 0.2-0.4%). Enzyme-to-substrate ratio should be ~1:50, but can be adjusted up/down to optimize digestion of different samples. Cap with short "150 µl" MicroCap.
14. Perform trypsin digestion in Barocycler at 52^0 C for 60-90 cycles at 20 or 45kpsi (50 sec at high pressure, 10 sec at ambient pressure, per cycle).
15. Proceed to sample clean-up using standard methods.

Materials and Methods

Lys-C (mass spectrometry grade) was purchased from Wako. Trypsin (sequencing grade) was purchased from Promega. Bovine serum albumin (BSA, fraction V), equine myoglobin and ubiquitin, were purchased from Sigma. Proteins were dissolved in 50mM ammonium bicarbonate and digested without additional processing. Deoxycholate (sodium salt) was from Amresco. Rat liver was purchased from Pel-Freeze. PCT was performed in a 2320EXT Barocycler, equipped with built-in temperature control. Digest reactions were carried out in 100mM ammonium bicarbonate (ambic) supplemented with the indicated concentration of Deoxycholate (DOX) or urea.

Tissue protein extraction was carried out using the standard MicroPestle protocol [1, 2] using 1-2mg of tissue per sample, in 30µl of the indicated lysis reagent. Each individual tissue sample was weighed prior to extraction. Pressure cycling was carried out at 35kpsi for 60 cycles (20 sec at high pressure, 10 sec at ambient pressure, per cycle) at ambient temperature. Protein yield was measured by BCA assay. Total yield is expressed as µg of protein recovered per mg of tissue wet weight, to normalize for differences in starting tissue mass.

Model protein digestion by Lys-C and trypsin was assayed using native BSA (disulfide-intact), myoglobin and ubiquitin as substrates. Pressure cycling 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 and temperature. Control reactions were incubated in MicroTubes at the same temperature but without pressure. Additional trypsin controls were incubated under standard conditions (atmospheric pressure, 37^0 C) in a Thermomixer with shaking. Enzyme-to-substrate ratio was 1:50 for all digests. 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 bands.

References

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