The Effect of Pressure Cycling on Proteolytic Cleavage Efficiency, Reaction Time and Protein Sequence Coverage

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Overview

Purpose: Examine the effect of pressure cycling technology (PCT) on proteolysis cleavage efficiency, reaction time, and protein sequence coverage for LC-MS/MS analyses.

Methods: Protein samples were digested with proteases under pressure cycling conditions and under standard digestion conditions. The resulting peptides were analyzed using LC-MS/MS with an ion trap mass spectrometer (MS) equipped with electron transfer dissociation (ETD) or an ion trap-Orbitrap hybrid MS.

Results: PCT digestion protocols using trypsin, chymotrypsin, LysC and GluC provided comparable sequence coverage to standard overnight digests. PCT digestion of complex mixtures of cytosolic proteins produced similar results. PCT improved the digestion of post-nuclear membrane proteins.

Introduction

Many methods have been used to enhance proteolysis cleavage efficiency, reaction time, and protein sequence coverage for LC-MS/MS analyses. The experiments presented here examine the effect of pressure cycling on these factors(1,2). Pressure cycling technology applies alternating hydrostatic pressure between 1 atm and high pressure to control molecular interactions. Several individual proteins and a mixture of 9 protein standards were digested with different proteases under high pressure using a Barocycler NEP2320 pressure cycler. These were compared to overnight digestion of the same proteins at 1 atm and 37 °C. The resulting peptides were identified and quantified using an ion trap equipped with ETD.

The studies were extended using an ion trap-Orbitrap hybrid MS to evaluate performance in the context of a complex cell model system: proteins extracted from human monocyte-like U937 hystiocytic lymphoma cells. This provided an opportunity to evaluate the overall performance of pressure cycling for complex mixtures. Additional experiments were conducted to examine the benefits of PCT for sequencing membrane proteins and also to examine the reproducibility of generating peptides with missed cleavages, a potential benefit for ETD-based detection.

Methods

An equimolar mixture of 9-protein standards (horse apomyoglobin, BSA, chicken egg lysozyme, bovine carbonic anhydrase, bovine α-lactalbumin, rabbit GAPDH, chicken egg ovalbumin, horse cytochrome C, and bovine beta-casein) were reduced and alkylated using standard protocols. Samples were digested in 100 mM ammonium bicarbonate (0.46 M in residual urea) at the pH appropriate for each protease and a concentration of 6 ng/µL (100:1 w/w substrate:enzyme): trypsin (pH 8.25), LysC (pH 9.2), GluC (pH 7.8) and chymotrypsin (pH 8.2). Individual-protein digests were performed for horse apomyoglobin (HMb), bovine carbonic anhydrase II (CAII), and reduced-alkylated BSA using 2 pmol/µL protein substrate and 6 ng/µL trypsin. Control digests were performed at 37 °C for 16 hours without mixing. All PCT digests were performed with a Barocycler NEP2320 pressure cycler. For all samples, the pressure cycle was set to ramp to 20 kpsi for 50 seconds followed by 10 seconds at 1 atmosphere at 50 °C. Cycles were repeated 30 times for the 9-protein sample and 20 times for the single proteins. Each single protein sample was subsequently spiked with human angiotensin II for peak area normalization.

For each experiment, undiluted sample was injected directly on to a Micro-Tech Scientific C-18 (100 x 0.150 mm) column. Samples of the 9-protein mixture were eluted with a 60-minute 0-60% ACN gradient and eluting peptides monitored with a Thermo Scientific LTQ XL with ETD using a top-three alternating CID/ETD method. Samples of individual protein digests were eluted with a 40-minute 0-80% ACN gradient and elution peptides monitored with an LTQ XL™ with ETD using a Triple-Play experiment with alternating CID/ETD. Three back-to-back replicate chromatographic runs were preformed for each sample.

U937 cell lysate and J774 post-nuclear supernatant (PNS) membrane protein aliquots were subjected to either overnight digestion at 37°C at 600 rpm and 1 atm, or in a Barocycler at 20 kpsi for 50 s followed by 10 s at 1 atm. Samples were acidified with 0.2% formic acid. The samples were loaded onto an home-made Jupiter C-18 precolumn (5 x 0.360 mm), eluted, and separated on an home-made Jupiter C-18 analytical column (100 x 0.150 mm) with a 56-minute 0-60 % ACN gradient with Eksigent 2D nano pumps. Eluted peptides were monitored and sequenced on a Thermo Scientifc LTQ Orbitrap XL hybrid MS with CID. Peptide coordinates (*m/z*, charge, retention time, and peak intensity) were determined with in-house MassSense software.

Results

- PCT produced higher % sequence coverage than the control for almost all proteins substrates and proteases examined (Figure 1).
- PCT produced higher numbers of distinct peptides than the control for all proteases examined (Figure 2).
- PCT produced more peptides with missed cleavages than the control for all proteases examined (Figure 3, panel A).
- PCT produced more missed cleavages per peptide than the control for all proteases examined (Figure 3, panel B).

FIGURE 1: Average % sequence coverage for individual proteins for proteolytic digests of a 9-protein mixture. Vertical lines represent ± 1 standard deviation.

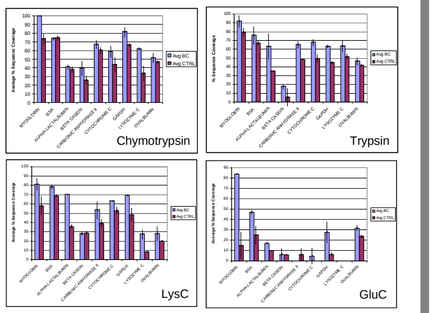


FIGURE 2: Average number of distinct peptides for proteolytic digests of a 9-protein mixture.

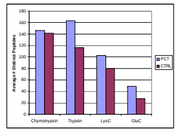
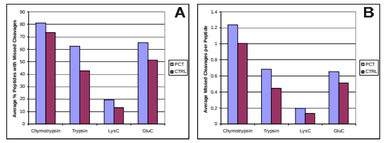
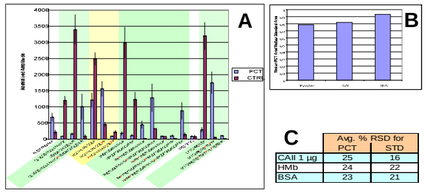


Figure 3: Average % of peptides with missed cleavages and average missed cleavages per peptide for proteolytic digests of a 9-protein mixture.



- In the single-protein experiments, PCT reproducibly produced missed cleaved peptides in larger numbers than the control (Figure 4, panel A).
- PCT reproducibly produced more peptides (Figure 4, panel C), but with generally lower amplitude (peak area) as shown in Figure 4, panel A.
- PCT produced overall less quantifiable peptide peak area than the controls (8-20%) as shown in Figure 4, panel B.

FIGURE 4: Reproducibility of missed cleavages and quantification of peptides from single-protein trypsin digests for both PCT and control (CTRL). A: Normalized peak areas for all quantifiable peptides obtained from horse apomyoglobin. Vertical lines represent ± 1 standard deviation. B: Ratio of PCT median peak areas to median control peak areas. C: Average % RSD for peptide peak areas.

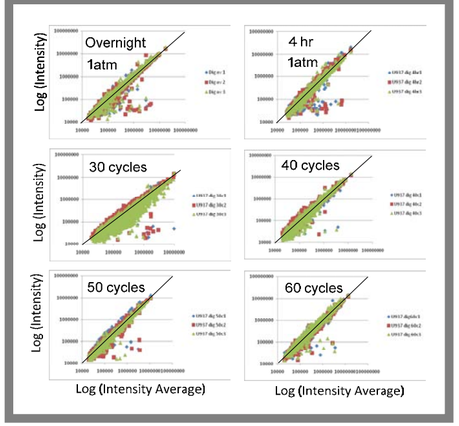


C

	Avg. % RSD for PCT	STD
CAII 1 µg	25	16
HMb	24	22
BSA	23	21

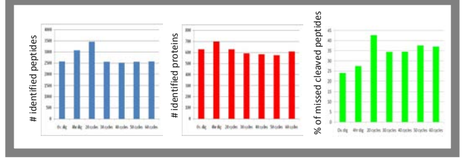
- In analyses of the U937 protein extracts, PCT produced lower abundance peptides less reproducibly than standard methods when 20-30 cycles were used (average RSD: 28%) as shown in Figure 5.
- Increasing the number of PCT cycles to 40 or more increased reproducibility of lower abundance peptides (average RSD: 16%).

FIGURE 5: Reproducibility of peptide intensities for trypsin digestions of U937 protein extracts under control and PCT conditions.



- More peptides were identified using 20-cycle PCT or 4-hr standard digestion.
- The higher number of peptides identified by 20-cycle PCT was due to the higher proportion of peptides with missed cleavages.
- Numbers of protein identifications were roughly equivalent.
- The missed-cleavage trend leveled off at 30 cycles.

FIGURE 6: Peptide identifications, protein identifications and missed cleavages for trypsin digests of U937 monocyte cell lysates



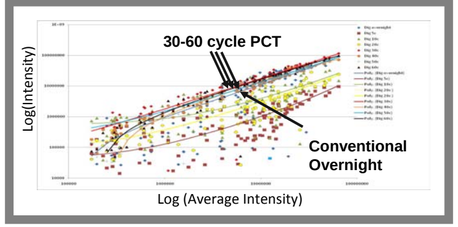
- PCT digestion produced equivalent numbers of peptides and better reproducibility than 4-hr standard digests without mixing (Figure 7).
- Increasing mass transfer capabilities of PCT digestions should markedly improve the numbers of peptide and protein identifications.

FIGURE 7: Comparison of PCT digestion to 4-hr control digestion without mixing.

	Thermomixer	Barocyler
# peptides	972	986
# proteines	324	302

- PCT using 30 or more cycles produced peptides in higher abundance than conventional overnight digestion methods (Figure 8).

Figure 8: In-gel digestion of BSA using PCT and conventional overnight digestion.



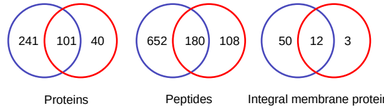
- PCT enabled a 2.5-fold increase in peptide identification compared to the conventional digestion procedure for post-nuclear membrane samples (Figure 9).
- PCT was also more effective in the digestion of integral membrane proteins.

FIGURE 9: Comparison of PCT and CTRL for post-nuclear membrane tryptic digests. The PCT digestion were 60 cycles of 50 s at 20 kpsi followed by 10 s at 1 atm.

A

	Barocycler	Thermomixer
Unique Peptides	832	288
Unique Proteins	342	141
Integral Membrane Protein	62	15

B



Conclusion

- PCT digestion protocols using trypsin, chymotrypsin, LysC and GluC have been established and provided comparable sequence coverage to standard overnight digests.
- PCT digestion of complex or simple protein mixtures reproducibly yielded a higher number of missed cleavages, a potential benefit for ETD ion activation.
- PCT with 60 cycles provided reproducible peak intensities (RSD: 16%) comparable to either standard 4-hour or overnight digestions (RSD: 15 %).
- PCT digestion of complex mixtures (U937) produced a comparable number of identified proteins to that from standard 4h or overnight digestions.
- Using 40 or more cycles improved reproducibility of PCT digestions of complex mixtures (U937) and provides more efficient proteolysis compared to conventional overnight digestion.
- PCT digestions with agitation should improve the number of identifications.
- PCT improved the digestion of post-nuclear membrane proteins and allowed the identification of a higher number of integral membrane proteins.

References

1. López-Ferrer D, Petritis K, Hixson KK, Heibeck TH, Moore RJ, Belov ME, Camp DG 2nd, Smith RD *J. Proteome Res.* (2008) 7(8), 3276-3281..
2. Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT *J. Biomol. Tech.* (2006) 17(2) 173-175.

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