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Application of Pressurized Solvents for Ultra-Fast Proteolysis: Proteomics on the Fly

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Complete trypsin digestion of proteins in 60 s

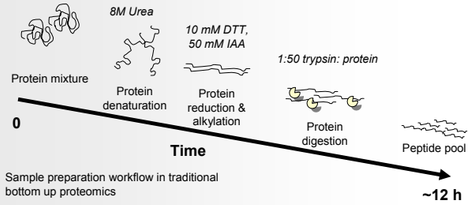
Overview

- The use of pressurized systems for protein digestion in proteomics was demonstrated.
- The performance of the different pressurized systems, pressure cycling technology (PCT) and fast on-line digestion system (FOLDS), was characterized.
- Digestions were accomplished in seconds without increasing the temperature.
- The coupling of FOLDS with IMS-MS allows complete protein characterization in < 90 s from injection to data output.

Introduction

- In current proteomic workflows, digestion is one of the most time consuming steps in the analytical process (i.e., incubation periods on the order of 3-12 h).
- Microwave and ultrasonic-assisted digestions have been shown to accelerate enzymatic reactions as a result of increased temperature and pressure.
- High pressure processing has been used in the food industries to accelerate enzymatic reactions.
- In proteomics, enzymatic reactions are used to digest proteins prior to analyses.
- Trypsin is the most commonly used enzyme in shotgun proteomics approaches.
- In this study, we evaluated pressures up to 35,000 psi as a means of accelerating tryptic digestions.

Methods



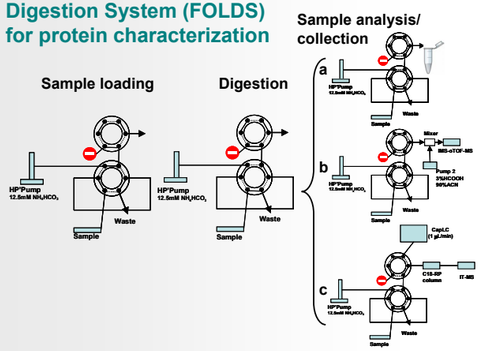
Sample preparation workflow in traditional bottom up proteomics

Pressure cycling technology for rapid protein digestions

The effect of pressure on a trypsin digestion was investigated off-line using BSA as the substrate and the Barocycler™ operating at different constant pressures (5, 10, 20 and 35 kpsi), as well as alternating pressures (cycling between ambient and high pressure, 0 to 35 kpsi).

The total digestion time was kept constant at 60 s. Protein digests were subsequently analyzed by LC-MS/MS.

Fast On-line high pressure Digestion System (FOLDS) for protein characterization

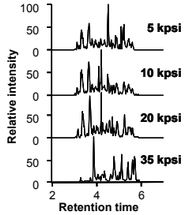


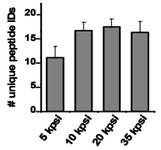
FOLDS in different configurations. The components of the system include a syringe pump, and two six port injection valves. The valves are shown in the positions corresponding to the stage of the experiment. **a)** Off-line set-up used with FTICR MS. **b)** FOLDS coupled to an IMS-o-TOF MS instrument. A secondary syringe pump was needed to re-acidify the sample prior to MS analysis. **c)** FOLDS coupled to an LC-MS system allowing peptide separation before detection. Proteins are injected onto the system with the necessary amount of trypsin and then pressurized in the loop for 1 min.

Results

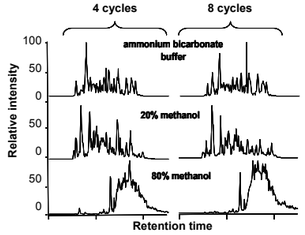
Effect of pressure on protein digestion

LC-MS/MS chromatograms of high pressure assisted digestions as a function of digestion pressure. High pressure in-solution digestions of BSA were performed at 5, 10, 20, and 35 kpsi.

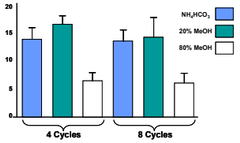




Histograms depicting the number of unique identified peptides for each method.

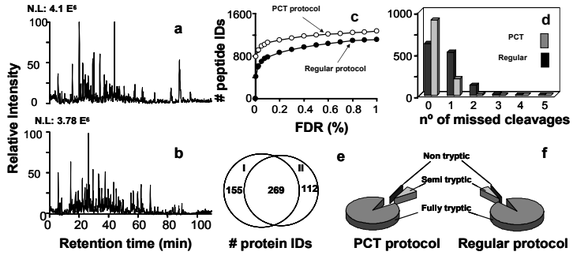


Chromatograms corresponding to the LC-MS/MS of PCT assisted digestion performance as a function of the number of cycles and the organic content in the digestion buffer.



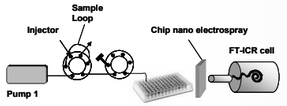
Unique peptides identified as a function of the number of pressure cycling technology (PCT) cycles performed at 35 kpsi and with different organic solvent compositions.

Application of PCT for digestion of a complex protein cell extract

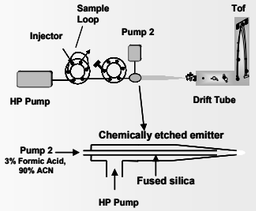


Comparison of a traditional digestion protocol versus a pressure-assisted digestion using *Shewanella oneidensis* as a model proteome. **a)** and **b)** Chromatograms obtained from LC-MS/MS analysis of traditional and PCT digestions, respectively. **c)** Number of identified peptides in both samples at a given FDR following SEQUEST analysis. **d)** Histogram showing number of identified peptides with missed cleavages. **e)** Overlap between the identified proteins. **f)** Comparison of the different identified peptides in terms of trypsin specificity.

FOLDS configurations

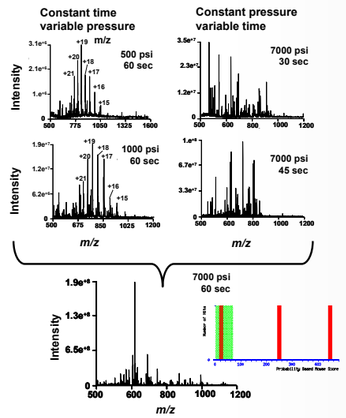


Experimental setup for FOLDS coupled off-line to a 12T FTICR MS.



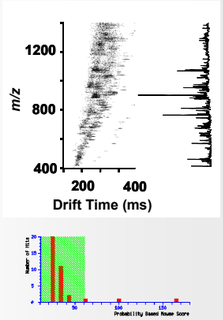
Experimental setup for on-line coupling of the FOLDS to IMS-oTOF MS. Lower schematic: Diagram of the mixing of aqueous and organic buffer solutions at the ESI tip.

System characterization



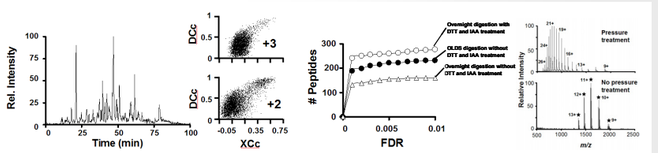
Sample loop was loaded with myoglobin and trypsin and then pressurized. Digested samples were directly analyzed using a 12T FTICR mass spectrometer. Right: Mascot peptide fingerprinting results for a 1 min digestion at 7000 psi.

Application to protein mixtures



Top: IMS-TOF MS map and the summed mass spectrum obtained with an equimolar mixture of α-lactoglobulin and myoglobin. Bottom: Mascot peptide fingerprinting results.

On-line application of FOLDS coupled to RPLC-ion trap MS



Left: BPC obtained in LC-MS/MS of *S. oneidensis* proteome after a 1 min digestion with FOLDS. Right: Distributions of the SEQUEST corrected scores for charge states 2+ and 3+.

Comparison of the number of identified peptides in the three analyzed samples at a given false discovery rate following SEQUEST analysis.

ESI mass spectra of horse myoglobin dissolved in 12.5mM ammonium bicarbonate.

Conclusions

- Pressure can play an important role in enzyme kinetics.
- The use of high pressure in protein tryptic digestion allows for short reaction times (1-2 min) without losing selectivity.
- High pressure helps to denature proteins, providing the enzyme better access for digestion.
- An on-line microreactor integrated into a capillary LC coupled online to MS has been developed and demonstrated.
- FOLDS coupled to IMS-TOF MS provides an analysis platform with the capacity to rapidly detect large numbers of peptide ions, which is useful for single protein characterization or monitoring.
- An automated version of FOLDS could prove valuable for ultra-fast high-throughput analyses or as a diagnostic/ clinical tool (e.g., quick screening or characterization of environmental samples; rapid and early detection of disease biomarkers in blood, urine, or saliva; etc.).
- We demonstrate for the first time the application of pressurized systems for complete protein digestion for LC-MS/MS proteomics experiments.

Acknowledgements

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