ACCELERATING DIGESTION OF PROTEOMES USING TRYPsin-IMMobilIZED MAGNETIC NANOPIRLES

Christie Hunter1, Uma Kota2, Sean Seymour1, Mark Stolowitz1
1AB SCIEX, USA; 2Canary Center at Stanford for Cancer Early Detection, Dept. of Radiology, Stanford School of Medicine, Palo Alto, CA, USA.

INTRODUCTION

Mass spectrometry (MS)-based protein characterization is typically performed using the trypsin digestion approach where proteins are subjected to enzymatic digestion or chemical hydrolysis to generate peptides. Reproducible, rapid and complete digestion of protein mixtures is crucial for in-depth protein identification and quantification in bottom-up proteomics. Conventional in-solution trypsin digestion is time-consuming and prone to artifacts, reducing dynamic range of MS detection in complex samples. Immobilized trypsin has several advantages, allowing higher protein to enzyme ratio, smaller sample volume, easy sample manipulation, limited autolysis products and shorter incubation times. Immobilized enzymes on magnetic particles offers a distinct advantage over soluble enzyme as they serve both as absorbers of microwave radiation that accelerates the digestion process and affinity to separate digestion products using an external magnetic field.

There are several different types of commercially available trypsin and immobilized digestion procedures for use in proteomics. The objective of this study is to evaluate the relative digestion efficiency and fidelity of the different types of trypsin modified, vaporized, microwave digestion techniques (in-solution, solid phase and microwave-assisted). In addition to measuring protein and peptide yield, the statistical analysis of the percentages of canonical sequences, missed cleavages and over-digestion products under different conditions allow a time course with a goal of finding the optimal conditions for quantitative MS studies.

The combination of the technologies employed here is particularly well suited to this type of assessment. First, the depth of acquisition provided by the TripleTOF™ 5600 System makes it much more likely to observe multiple copies of the desired canonical peptide, including those arising from low stoichiometry species. Secondly, the combination of the use of sequence tags in the sequence Tag Value approach and use of the Paragon™ Algorithm in ProteinPilot Software makes it very likely to look for large numbers of digestion and modification variations without the usual loss in identification discrimination. The ProteinPilot Descriptive Statistics Template enables easy dissection of the massive amounts of identification data into informative tables.

METHODS

Preparation of Trypsin Immobilized Magnetic Particles (TIMPs): Unmodified porcine pancreatic trypsin (USBioAnalyzed/Affymetrix) was immobilized onto magnetic beads,1 µm; BiocloneInc.) by cross-linking the primary amines of trypsin with 1% N-octylglucoside(OGS) from products by using an external magnetic field.

Comparison of Different Digestion Strategies: E. coli cell lysate was denatured using 1% N-octylglucoside(OGS) and diluted with 100X heated 500 mM TCEP and aliquoted at 1:20 for each 4 time points. Solid-phase digestion was done using 300 μg of TIMPs at 50% E/S of lysate at 60°C for 120 min and in-solution digestion was carried out using a domestic microwave with output power of 1200 W.

Comparison of Different Types of Denaturants and Trypsin 203T cell lysate was denatured using 1% N-octylglucoside(OGS) or 0.1% Rapigest followed by reduction and alkylation as above. In solution digestion was done using Protease Sequence Grade Modified Trypsin (Trypsin 1) or Sigma Protamex Proteases (Trypsin 2) at an E/S of 1:20 at 37°C. All solid-phase and solid-phase + microwave digestion time points used in this study.

Chromatography: The sample was analyzed using the Agilent Nano-Ultimate 2100 System Plus under the same conditions as discussed above (Eksigent, Dublin, USA). The cell lysate (1 ul total protein) was loaded at 2 μL/min over a 200 μm × 0.5 mm column and eluted at 300 nL/min with a flow rate of 0.1% acetonitrile/0.1% formic acid with a 60 min gradient was used.

Mass Spectometry: Data was acquired on the TripleTOF™ 5600 System in IDA mode using a 10% valve of 20 MS/MS per cycle with a minimum accumulation time of 0.1 sec. High sensitivity MS/MS mode was used as MS/MS spectra had resolution >15,000.

Data Processing: All data was processed using ProteinPilot™ Software 4.0, using the integrated false discovery rate analysis. Results were then analyzed using the PSPEP Comparison Template and the ProteinPilot Descriptive Statistics Template with some integrated false discovery rate analysis. Results were then analyzed using the PSPEP Comparison Template and the ProteinPilot Descriptive Statistics Template with some integrated false discovery rate analysis. Results were then analyzed using the PSPEP Comparison Template and the ProteinPilot Descriptive Statistics Template with some integrated false discovery rate analysis. Results were then analyzed using the PSPEP Comparison Template and the ProteinPilot Descriptive Statistics Template with some integrated false discovery rate analysis. Results were then analyzed using the PSPEP Comparison Template and the ProteinPilot Descriptive Statistics Template with some integrated false discovery rate analysis.

RESULTS

The relative efficiency of trypsin digestion under the many different conditions tested here was measured in terms of yield at the peptide and protein level, percentage canonical sequences, missed cleavages and over-digestion products as well as artifacts formed during the course of in-solution digestion.

Comparison of the two different trypsins relative to each other when the same enzyme was used shows very little difference between using RapiGest or OGS (RapiGest providing ~10% more peptides); however, comparing the two different types of trypsins shows that Trypsin + Microwave digestion gives a yield better compared to immobilized trypsin. The plot of peptide yields from solid-phase digestion methods could due to non-specific sample absorption and/or protein aggregation on the TIMPs.

Figure 1: Overview of Experimental Design. Analysis of all samples was performed in the same day and analyzed using trypsin digestion in the in-solution parameters that were varied.

Figure 2: Comparison of Yield from Different Digestion Strategies. 20 μg of E.coli cell lysate was denatured in 1% N-octylglucoside(OGS) and diluted with 500 mM TCEP and aliquoted at 1:20 for each 4 time points. Solid-phase digestion was done using 300 μg of TIMPs at 50% E/S of lysate at 60°C for 120 min and in-solution digestion was carried out using a domestic microwave with output power of 1200 W.

Figure 3: Comparison of Digestion Efficiency between Trypsin-treated and Unmodified Trypsin. Data was acquired on the TripleTOF™ 5600 System in IDA mode using a 10% valve of 20 MS/MS per cycle with a minimum accumulation time of 0.1 sec. High sensitivity MS/MS mode was used as MS/MS spectra had resolution >15,000.

Figure 4: Digestion Map Comparing the Cleavage Frequency by Residue Pair for the Different Grades of Trypsin. (A) Data is presented as a relative frequency of cleavages for E. coli cell lysate digested in solution with Trypsin-treated trypsin (A) or unmodified trypsin (B). Untreated trypsin has chymotrypsin activity that cleaves after Leu (L), Phe (F), Trp (W) and Tyr (Y) residues. The chymotrypsin activity in the untreated trypsin as seen in Figure 4[B] explains higher preference for lower cleavage observed in Figure 4[A].

Figure 5: Comparison of Different Digestion Reagents for Impact on Canonical Peptides. Modified trypsin from different vendors in combination with commonly used denaturants (OGS and RapiGest) was used to digest human cell lysates to understand the effect on the quality and efficiency of digestion. (Continued above)

Figure 6: Characterization of Sequencing Grade Protease Trypsin. Analysis of the protein yield and MS spectral analysis done in Figure 6(A) indicates that the Trypsin digestion does not remove the digestion artifacts and therefore may be less desirable than previously believed. A similar trend is seen for digests produced with in-solution digestion in Figure 6(B) and the percentage of peptides that are canonical(OGS) is plotted. As expected, there is a decrease in the fraction of peptides that are canonical as the less abundant proteins (measured as precursor signal) in the sample are analyzed.

CONCLUSIONS

• Multiple digestion strategies and reagents were evaluated for their yield and fidelity.
• Analysis of digestion time courses concurrent with the analysis of canonical peptides and their under- and over-digested counterparts is key to understanding and optimizing digestion for quantitative MS applications.
• The combination of deep acquisition with the TripleTOF™ 5600 system and extensive identification provided by the analysis with the Paragon™ Algorithm allowed this level of interrogation in a way that has not been possible before.
• Use of Trypsin Immobilized Magnetic Particles (TIMPs) and the combination with microwave digestion shows a loss in peptide yield possibly due to surface digestion artifacts and therefore may be less desirable than previously believed.
• Protease grade trypsin from Sigma showed a higher rate of missed cleavages across the time points suggesting that perhaps this enzyme had lower activity or required a higher protein to enzyme ratio (needs to be investigated).
• N-Octylglucoside and RapiGest provided similar profiles in terms of canonical peptides and their under- and over-digested counterparts is key to understanding and optimizing digestion for quantitative MS applications.

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