Comparison of Immobilized Protease and In-Solution Approaches for MS Sample Preparation

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Overview

Purpose: To evaluate an immobilized trypsin-digestion workflow for improved proteomic sample preparation.

Methods: Two different methacrylate-based monolithic tips, IMT tips and Diges Tips, with covalently-bound trypsin were evaluated for protein digestion. Mass spectrometry analyses was performed using hybrid quadrupole-Orbitrap and ion trap mass spectrometers.

Results: We observed comparable sequence coverage for a single protein digested in solution (4 hours) or on tips (10 min). For LysC pre-digested HeLa cells, a 20 min digestion on immobilized-trypsin monolithic tips produced only 20% less protein identification than a 3 hr digestion in solution.

Introduction

Reproducible and efficient protein digestion is the critical step in the identification and quantitation of proteins by bottom-up mass spectrometry. A combination of LysC and trypsin proteolytic digestion, performed in solution, is often used to improve protein sequence coverage. However, this approach can result in extended incubation times, low efficiency, and enzyme auto-digestion. To overcome these limitations, we compared the performance of four different immobilized trypsin pipette tips and traditional in-solution digestion, for the digestion of purified proteins and LysC pre-digested complex protein samples by MS analysis.

Methods

Sample Preparation

HeLa cell lysate, recombinant green fluorescent protein (GFP), and in vitro expressed heavy and light isotope-labeled Bcl-2-associated death promoter (BAD) proteins were supplied by Thermo Fisher Scientific. HeLa proteins were reduced, alkylated and subjected to a 2 hr LysC digestion in solution. Lys-C predigested HeLa samples were then digested with trypsin in solution or using immobilized-protease tips. Two different methacrylate-based monolithic tips, IMT tips (University of Tasmania, Australia) and Diges Tips (Siena, Italy), with covalently-bound trypsin were used.

LC-MS

A Thermo Scientific EASY-nLC™ 1000 HPLC system and Thermo Scientific EASY-Spray Source with Thermo Scientific Dionex Acclaim Pepmap™ C18 15 cm x 75 μm i.d. column was used to separate peptides with a 5-40% acetonitrile gradient in 0.1% formic acid over 30 min (single protein) or 90 min (HeLa lysate) at a flow rate of 300 nL/min. The samples were analyzed using Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap or Thermo Scientific Velos Pro ion trap mass spectrometers.

Data Analysis

Thermo Scientific Proteome Discoverer software version 1.3 was used to search MS/MS spectra against the IPI human database (HeLa cell lysate) or custom generated 20 protein database (recombinant purified proteins) using either SEQUEST® or Mascot™ search engines. Static modifications included carbamidomethyl (57.02 Da) and methionine oxidation. Dynamic modifications included serine, threonine, and tyrosine phosphorylation. Database search results were imported into Thermo Scientific Pinpoint software version 1.2 to perform HR/AM MS-level quantitation using the Q Exactive™ instrument data. Data extraction was based on the three most abundant isotopes per charge state, per targeted peptide. The area-under-the-curve (AUC) values were summed to obtained total AUC values. The relative AUC values for each of the isotopes were compared against the theoretical isotopic distribution for confirmation.

FIGURE 1. Schematic diagram showing in situ preparation of polymer monoliths in polypropylene pipette tip (A) and two models of through-channel tips (B).
Results

Comparison of Single-Protein Digestion In-Solution and Using Immobilized Trypsin Tips

In this study, we used 100 µL Diges Tips (ProteoGen Bio, Italy) and two different types (with or without through-channel) of immobilized-trypsin monolithic (IMT) tips as shown in Figure 1A. We evaluated two models of through-channel monolithic tips: (a) monolithic bed in the middle of the tips (IMT-a); (b) monolithic bed at the end of the tip (Figure 1B).

First, we evaluated all types of immobilized-trypsin tips for digestion of Thermo Scientific recombinant green fluorescent protein (MW = 27.5kDa), Product No. 88899. Experimental conditions are summarized in Table 1. Sequence coverage results for the GFP using soluble trypsin, immobilized-trypsin monolithic (ITM) tips and 100 µL Diges Tips are shown in Figure 2.

For the single protein digest, we used 50 mM ammonium acetate buffer at two different pH, pH 8 (Figure 2A) and 8.6 (Figure 2B), and two different sample loads, 25 µg (A) and 25 ng (B). We observed no difference in sequence coverage of a single protein digested in solution (4 hours) or on immobilized trypsin tips (10 min) at both high and low sample loads. All types of immobilized trypsin tips performed equally well and were reproducible (<15%). We found that pH 8 is optimal for on-tip digestion. At the higher pH we observed multiple trypsin autolysis peaks (Figure 3) and reduced sequence coverage (Figures 2A versus 2B). We obtained similar results for in vitro expressed heavy- or light isotope-labeled BAD protein (MW =18kDa).

TABLE 1. GFP digestion conditions for Figure 2.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Trypsin</th>
<th>IMT tips</th>
<th>DigesTips</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample volume</td>
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<td>50 µL</td>
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<tr>
<td>Sample concentration</td>
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<td>0.5 ng/µL(B)</td>
<td>0.5 ng/µL(B)</td>
<td>0.5 ng/µL(B)</td>
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<td>Equilibration volume</td>
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<td>500 µL</td>
<td>500 µL</td>
</tr>
<tr>
<td>Incubation</td>
<td>0 min</td>
<td>20 min</td>
<td>0 min</td>
</tr>
<tr>
<td>Digestion</td>
<td>4 hours</td>
<td>10 min</td>
<td>2-5 min</td>
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</table>

FIGURE 2. Percent GFP sequence coverage after digestion in 50 mM Ammonium acetate, pH 8.0, 20% ACN with 1 pmol/injection (A) or 50 mM Ammonium acetate, pH 8.6, 20% ACN with 80 pmol/injection (B).

A. 1 pmol injection

B. 80 fmol injection
Comparison of Immobilized Protease and In-Solution Approaches for MS Sample Preparation

HeLa Cell Lysate LysC+Trypsin Digestion

Multiple enzymes are often used in combination to improve sequence coverage on the three most abundant isotopes per charge state, per targeted peptide. The area compared the performance of four different immobilized trypsin pipette tips and low efficiency, and enzyme auto-digestion. To overcome these limitations, we trypsin proteolytic digestion, performed in solution, is often used to improve protein reproducibility and efficient protein digestion is the critical step in the identification and analyses was performed using hybrid quadrupole-Orbitrap and ion trap mass spectra against the IPI human database (HeLa cell lysate) or custom. Dynamic modifications included carbamidomethyl (57.02 Da) and methionine oxidation. Static modifications included spray source with Thermo Scientific Dionex Acclaim® Pepmap™ C18 15 cm x 75 μm. A Thermo Scientific EASY-nLC™ 1000 HPLC system and Thermo Scientific EASY-MS/MS spectra against the IPI human database (HeLa cell lysate) or custom.

In situ preparation of polymer monoliths and in vitro expressed heavy- or light isotope-labeled BAD protein (MW = 18kDa). the higher pH we observed multiple trypsin autolysis peaks (Figure 3) and reduced protein digested in solution (4 hours) or on immobilized trypsin tips (10 min) at both digestion on immobilized-trypsin monolithic tips produced only 20% less protein compared to digestion in solution. We observed comparable sequence coverage for a single protein digested in solution IMT IMT- a DigesTip digestion reproducibility one is the most important parameters in quantitative proteomics. To evaluate on-tip digestion reproducibility, we spiked 20 fmol/µL of GFP into produced the lowest number of identifications due to its limited capacity. peptides with missed cleavages and/or longer peptides which were not observed in the digestion in-solution for protein identification (Figure 6A); however, in-solution digestion this information is not intended to encourage use of these products in any manners that might infringe the

References

2. Wisniewski, J.R, Zougman, A., Mann, M. Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane.

FIGURE 1. Schematic diagram showing distribution for confirmation. The area

FIGURE 3. Base peak LC/MS chromatograms for GFP digestion at pH 8.6, sample concentration 0.5 ng/µL, 80 fmol injection. T = trypsin auto-digestion peaks.

FIGURE 4. HeLa cell lysate preparation

HeLa Cell Lysate +LysC 2 hrs at 37°C

LysC HeLa cell digest, 50 ng/µL + GFP, 0.5 ng/µL in 50 mM NH4acetate, pH 8.6, 20% AcN

FIGURE 5. Base peak LC/MS chromatograms for HeLa/GFP digestions prepared as in Figure 4. Arrows mark GFP peptides used for reproducibility estimation.
Overall, a 10 to 20 min on-tip digestion was comparable in performance to a 3 hr digestion in-solution for protein identification (Figure 6A); however, in-solution digestion outperformed both IMT (20% fewer total identified peptides) and a 100 µl DigesTip (40% fewer total identified peptides) for peptide identification (Figure 6B). This result may be attributed to the LysC-trypsin-tip combination which generated significantly more peptides with missed cleavages and/or longer peptides which were not observed in the solution digest (Figure 7). These observed differences in the peptide populations may be due to distinct proteolytic mechanisms or to non-optimal conditions for on-tip digestion. In addition, we used two different pH (7 and 8.6) and sample loads (50 µg and 5 µg); however, there was no improvement to overall results. Moreover, the 10 µL Diges Tip produced the lowest number of identifications due to its limited capacity.

Digestion reproducibility one is the most important parameters in quantitative proteomics. To evaluate on-tip digestion reproducibility, we spiked 20 fmol/µL of GFP into each complex protein sample. Based on spiked GFP sequence coverage (Figure 8), total area (Figure 9) and individual peptide (highlighted in Figure 5) and quantitation (Figure 10), both types of tips demonstrated reasonable reproducibility (< 10-20% CV).

**FIGURE 6.** Identification results comparison for in-solution and on-tip digestion: protein groups (A) and unique peptide (B) at 1 % FDR. Results represent an average of duplicate runs for each sample.

**FIGURE 7.** Peptide missed cleavage distribution after trypsin digestion.

**FIGURE 8.** Sequence coverage of GFP spiked in HeLa LysC digest.

**FIGURE 9.** Reproducibility of triplicate on-tip digestions of GFP spiked into the LysC HeLa cell digest. Graphs show total area of signal from all quantified GFP peptides for IMT (A) and Diges Tip (B) tips.
FIGURE 1. Schematic diagram showing identification on the three most abundant isotopes per charge state, per targeted peptide. The area-...

Conclusions

- Immobilized monolithic trypsin (IMT) tips can increase sample preparation throughput with fast digestion kinetics for purified proteins.
- Digestion bed formulation and capacity are important requirements for achieving efficient digestion.
- Reproducibility of on-tip digestion for both the single and complex protein mixture was better than 20%.
- Compared to in-solution digestion, both the digestion efficiency and sequence coverage of complex protein samples are lower when on-tip digestion is used. However, on-tip digestion is fast and reproducible.

References


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