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TECH NOTE

Fast, simple, and efficient protein digestion with Capturem Trypsin technology

Provides rapid and complete protein digestion >>

Digests tightly folded proteins >>

Generates peptides with high sequence coverage >>

Suitable for proteomic analysis of whole cell lysates >>

Introduction

Mass spectrometry (MS) is the main analytical technique used in proteomics and characterization of proteins from a wide variety of organisms and cell types. The most important step in MS analysis is sample preparation, which requires the conversion of proteins to peptides. Trypsin is the most commonly used enzyme for proteolytic digestion of proteins to prepare peptides for analysis. It is a highly specific serine protease that cleaves at the carboxyl side of lysine and arginine residues, generating peptides of optimal size for MS analysis. Rapid and complete digestion is important for detailed protein characterization.

The current practice in sample preparation is in-solution trypsin digestion for up to overnight at 37°C. Prolonged digestion of proteins may lead to oxidation or other protein modifications, resulting in poor sensitivity, thus making in-solution digestion less reproducible and unsuitable for automation. In addition, in-solution trypsin digestion fails to digest proteins completely, compromising protein identification in MS analysis.

Capturem Trypsin provides rapid, efficient, and complete digestion of protein samples, allowing an uninterrupted MS workflow at room temperature for downstream protein analysis. This product utilizes our novel Capturem technology in a spin column format with membrane-immobilized trypsin. Capturem Trypsin Columns may be used to completely digest protein samples in less than a minute with digestion efficiencies (protein coverage) comparable to or better than those obtained using in-solution trypsin digestion. Rapid protein digestion not only saves time but also improves peptide and protein identification in proteomic analysis by reducing trypsin autolysis.

Results

Rapid and complete protein digestion using Capturem Trypsin

Using apomyoglobin (Apo), a common standard for peptide mapping, we compared the efficiency of in-solution trypsin digestion to the Capturem Trypsin method. Apo lacks disulfide bonds, so it does not require denaturation and/or reduction before enzymatic digestion. The completeness of tryptic digestion is often used as a measure of digestion efficiency. Figure 1 shows that 100% of the Apo sample was digested with Capturem Trypsin following a single centrifugation at 500*g* for 1 min (Panel C), compared to only 70% of the Apo sample following 16 hr of digestion with in-solution trypsin, which displays a significant peak of intact Apo (Panel B).

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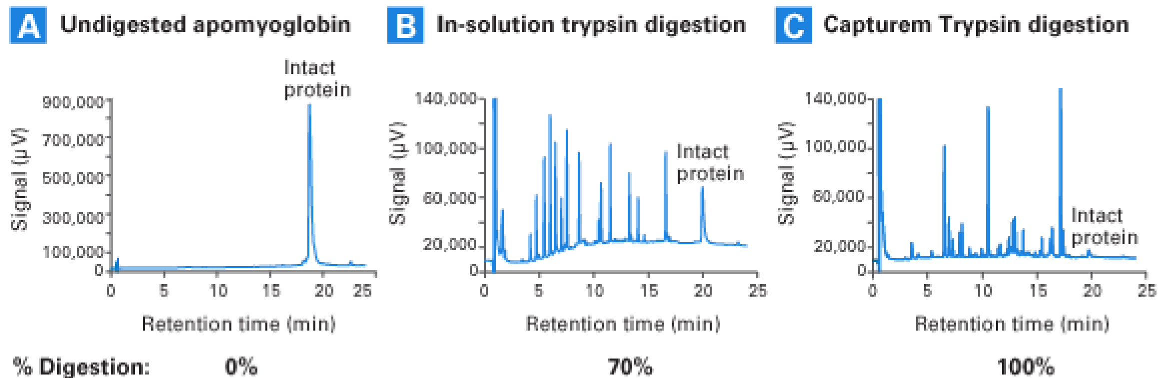

Figure 1. Rapid and complete digestion of apomyoglobin (Apo) using Capturem Trypsin. Reverse phase HPLC (RP-HPLC) analysis was used to compare undigested Apo (**Panel A**) with Apo digested using in-solution trypsin for 16 hr at room temperature (**Panel B**) and Apo digested on a Capturem Trypsin Column for less than 1 min (**Panel C**). Column activation, protein digestion and RP-HPLC analysis was carried out as described in the Methods section below.

Digestion of a tightly folded protein using Capturem Trypsin

Another limitation of in-solution trypsin digestion is its inability to digest highly folded proteins without additional sample processing steps such as denaturation and reduction. Myoglobin (Myo) is an example of a tightly folded globular protein that was poorly digested using in-solution trypsin. As shown in Figure 2, a Capturem Trypsin Column generated many proteolytic peptides from a Myo digestion in a single spin at 500*g* for 1 min (Panel C). In contrast, in-solution trypsin digestion for 16 hr (Panel B) failed to generate any proteolytic peptides and most of the Myo remained undigested.

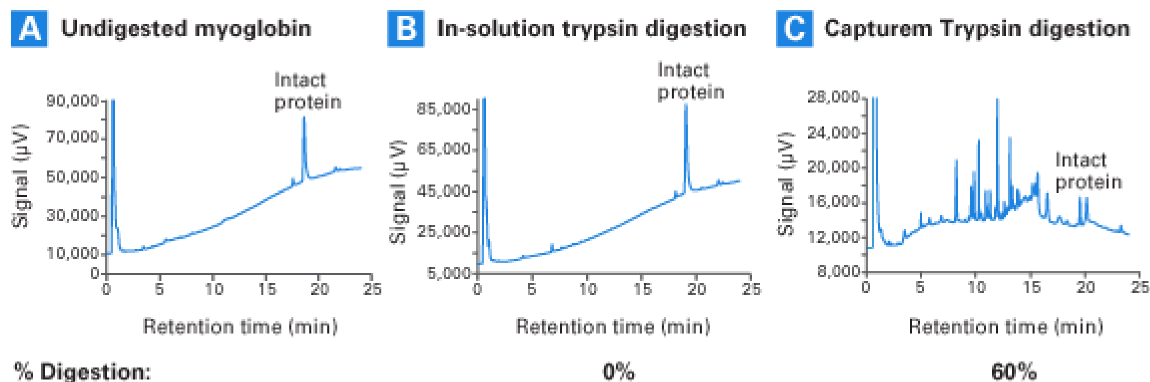

Figure 2. Proteolytic digestion of tightly folded myoglobin (Myo) using Capturem Trypsin. Reverse phase HPLC (RP-HPLC) analysis was used to compare undigested Myo (**Panel A**) with Myo digested using in-solution trypsin for 16 hr at room temperature (**Panel B**) and Myo digested on a Capturem Trypsin Column for less than 1 min (**Panel C**). Column activation, protein digestion and RP-HPLC analysis was carried out as described in the Methods section below.

Mass spectrometry analysis of a protein digested with Capturem Trypsin

In addition to determining the completeness of digestion, measuring the percentage of sequence coverage for the resulting peptides provides another way to evaluate tryptic digestion efficiency. To determine how effectively Capturem Trypsin generates peptides that provide good sequence coverage, we performed mass spectrometry (MS) analysis of Apo digested with Capturem Trypsin. Figure 3 shows the deconvoluted ESI-Orbitrap mass spectrum of Apo digested using Capturem Trypsin. A single centrifugation at 500*g* for 1 min provides complete digestion of Apo. MS analysis identified 26 Apo peptides, with seven of these peptides covering 100% of the amino acid sequence.

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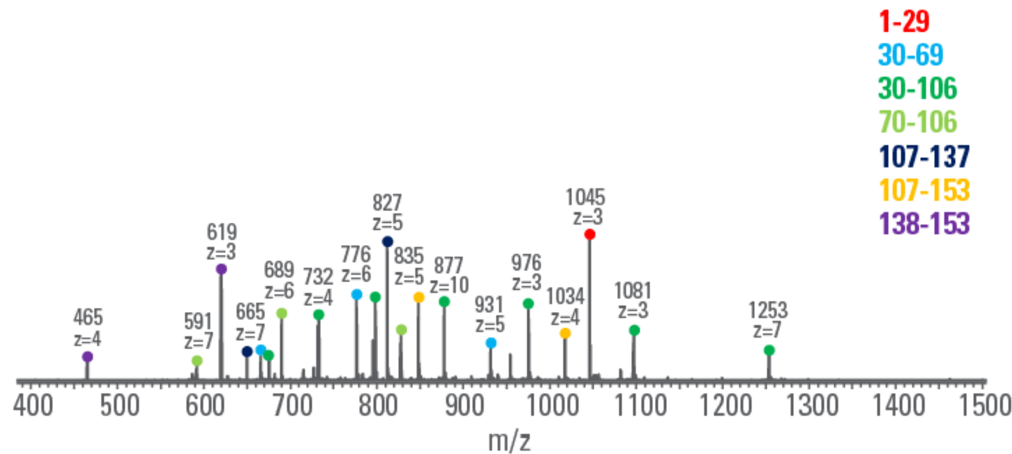

Figure 3. Mass spectrometry analysis of apomyoglobin (Apo) peptides generated by Capturem Trypsin. A deconvoluted ESI-Orbitrap mass spectrum was generated for Apo digested on a Capturem Trypsin Column, using Xtract software. Data kindly provided by: Dr. Merlin Bruening, University of Notre Dame.

Proteomic analysis of a whole-cell lysate digested with Capturem Trypsin

We used Capturem Trypsin for proteomic analysis of a whole-cell lysate made from BT474 ductal breast carcinoma cells, to identify as many unique proteins as possible. The denatured whole-cell lysate was fractionated by RP-HPLC (Figure 4, Panel A) into 19 separate fractions, each of which was digested with Capturem Trypsin. The amount of protein digested per fraction using Capturem Trypsin is provided in Table 1. The digested proteins in each fraction were analyzed in separate LC/MS-MS runs. Figure 4, Panel B shows the number of unique proteins identified per fraction. Overall, proteomic analysis of BT474 cells using Capturem Trypsin digestion identified a total of 2,320 unique proteins.

Table 1. Protein amount per fraction used for Capturem Trypsin digestion

Fraction	Protein amount (µg)
1	32.22
2	35.08
3	33.75
4	41.76
5	41.76
6	38.21
7	38.21
8	38.40
9	30.32
10	35.23
11	35.19
12	33.12
13	26.88
14	31.88
15	29.41
16	26.48
17	25.98
18	24.63
19	21.48

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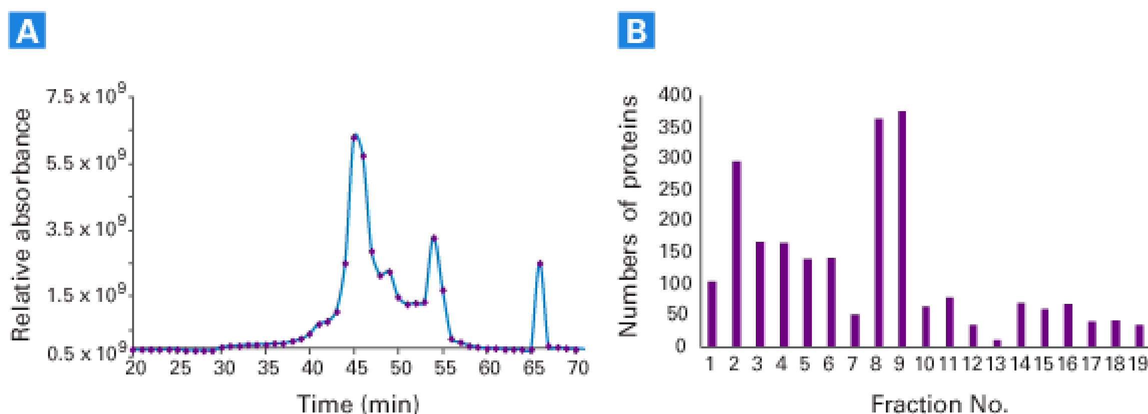

Figure 4. Proteomic analysis of BT474 whole-cell lysate digested using Capturem Trypsin. The lysate was fractionated using RP-HPLC (**Panel A**) into 19 separate fractions, which were then individually digested with Capturem Trypsin and analyzed by LC/MS-MS as described in detail in the Methods section below. The total number of proteins with two or more unique peptides was identified in each fraction as described in the Methods section and plotted on the graph (**Panel B**).

Conclusions

Capturem Trypsin provides faster and more complete digestion than in-solution trypsin digestion, preventing the loss of sensitivity and reproducibility than can result from prolonged and/or incomplete protein digestion. The superior speed and completeness of Capturem Trypsin digestion was demonstrated when both methods were used to digest apomyoglobin, a protein used as a standard model for protein mapping. Capturem Trypsin was also much more effective at digesting myoglobin, a tightly folded protein. Further analysis of a Capturem Trypsin digest of apomyoglobin using mass spectrometry confirmed that the peptides generated by the digest provide good sequence coverage. Capturem Trypsin digestion of a complex sample, a whole-cell lysate fractionated using RP-HPLC, enabled the identification of 2,320 unique proteins using LC/MS-MS analysis. These capabilities make Capturem Trypsin a powerful tool for proteomic analysis.

Methods

Apo and Myo digestion using Capturem Trypsin

Apo (80 µg) or Myo (10 µg) was dissolved in 200 µl of 10 mM ammonium bicarbonate before digestion. Capturem Trypsin Columns were activated by loading 200 µl of the activation buffer, included in the kit, onto the columns, followed by centrifugation at 500*g* for 1 min. The Apo or Myo solution was then loaded onto an activated column, followed by a single centrifugation at 500*g* for 1 min. The eluted peptides were subjected to downstream RP-HPLC and MS analysis.

Apo and Myo digestion using in-solution trypsin digestion

Apo (80 µg) or Myo (10 µg) was dissolved in 200 µl of 10 mM ammonium bicarbonate for in-solution trypsin digestion. Trypsin was used at an enzyme:substrate ratio of 1:20 (w/w) to digest 4 µg of Apo or 0.5 µg of Myo. The reaction mixture was incubated at room temperature for approximately 16 hr.

RP-HPLC analysis of digested Apo and Myo

RP-HPLC analysis was performed using a Rainin Dynamax HPLC system. Analysis was carried out at 215 nm on an Aeris 3.6 µm PEPTIDE XB-C18 100 Å, 50 x 4.6 mm column (Phenomenex) using gradient elution with two solutions: Solution A: 95% water + 5% acetonitrile + 0.1% TFA [v/v] and Solution B: 10% water + 90% acetonitrile + 0.085% TFA [v/v]. The following gradient program was applied: 95% A for 2 min, 5–60% B for 24 min, 60–70% B for 1 min, and 70% B for 2 min. A 5-min equilibration was performed with 95% A before injection. The injection volume was 20 µl.

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Mass spectrometry analysis of Apo digested with Capturem Trypsin

For direct infusion MS, the eluted peptides were dried with a SpeedVac and reconstituted in 1% acetic acid, 49% H_2O, and 50% methanol. Then 40 µl of reconstituted sample was loaded into a Whatman Multi-Chem 96-well microplate (Sigma-Aldrich) and sealed with Teflon Ultrathin Sealing Tape (Analytical Sales and Services). An Advion Triversa Nanomate nanoelectrospray ionization (nESI) source (Advion) was used to introduce the sample into a high-resolution accurate-mass LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) equipped with a dual pressure ion trap, HCD cell, and ETD. The spray voltage and gas pressure were set to 1.4 kV and 1.0 psi, respectively. The ion source interface had an inlet temperature of 200°C with an S-Lens value of 57%. High-resolution mass spectra were acquired in positive ionization mode across the m/z range of 300–1,800, using the FT analyzer operating at a mass resolving power of 100,000. Spectra were the average of 100 scans. Signals with >1% of the highest peak intensities and S/N>3 were analyzed. Peptide identification was performed manually using ProteinProspector (v 5.14.1, University of California, San Francisco). The analysis included maximum of 5 missed cleavages. Mass tolerance was set to 10 ppm. The mismatch was set to 2.

Proteomic analysis of BT474 whole-cell lysate

Proteomic analysis was performed using a whole-cell lysate made from BT474 ductal breast carcinoma cells in the laboratory of Prof. Kian Kani, University of Southern California, Los Angeles. Briefly, BT474 cells were lysed in PBS, 1% OG, with a full complement of protease and phosphatase inhibitors. The cells were then subjected to reduction (5 mM DTT) and alkylation (50 mM IAA) in a final concentration of 6 M guanidine-HCl in PBS/OG. Protein identification was carried out using liquid chromatography/tandem mass spectrometry (LC/MS-MS) in a data-dependent mode. To reduce sample complexity, an initial RP-HPLC protein fractionation was performed by injecting 2 mg of protein lysate (in 3M guanidine hydrochloride in PBS) and collecting 1-ml fractions every 30 sec, for a total of 19 separate fractions, which were then frozen, and dried with a SpeedVac. Each fraction was resuspended in 100 mM ammonium bicarbonate buffer, pH 8.0 containing 1 M urea + 1% acetonitrile (ACN), for a total volume of 200 µl. Each fraction was individually digested with a Capturem Trypsin Column as described in the Capturem Trypsin Protocol-At-A-Glance, and 2 µl of acetic acid was added to each column eluate to terminate the digestion reaction, which was was then cleaned up with a C18 ZipTip filter, frozen, and dried with a SpeedVac. Each fraction was then resuspended in 25 µl of 95/5/0.1 H_2O/ACN/FA (to an approximate concentration of 2 µg/µl). LC-MS/MS was performed by injecting 10 µl of each resuspended fraction into in an LTQ-FTICR or LTQ-ORBITRAP mass spectrometer (Thermo-Finnigan) coupled to a nanoflow chromatography system (Eksigent) with a 25-cm column (Picofrit 75 µm ID, New Objectives) packed in-house with MagicC18 resin (Michrom Bioresources), over a 90-min linear gradient. MS was performed on the 19 samples in a randomized order. Acquired data were automatically processed by the Computational Proteomics Analysis System. The tandem mass spectra were searched against Version 3.13 of the human International Protein Index database (60,428 protein entries) with 5 sequences for human and bovine trypsin added. The search was conducted with X!Tandem (2005.12.01). The mass tolerance for precursor ions was set during the search to 1 AMU, with a mass tolerance for fragment ions set to 0.5 Daltons. However, matches with less than 5 parts per million mass accuracy were considered false positives and discarded. All identifications with a PeptideProphet probability greater than 0.9 were submitted to ProteinProphet, and the subsequent protein identifications were filtered at a 1% error rate with tryptic fragments (2 missed cleavages) with allowance for fixed modification on C = 57.021 and variable modifications on C = –17.027, E = –18.011, K = 6.020, M = 15.995, and Q = –17.027. Proteins were identified by the presence of 2 or more peptides from each protein.

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