High Density Immobilized Trypsin in a Spin Column Format for Rapid Proteolysis and Increased Sequence Coverage

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Abstract

Tryptic digestion of proteins is one of the critical steps in any proteomics analysis and may heavily impact the quality of the data generated from the sample. The peptides generated are analyzed by mass spectrometry and used to identify and characterize the proteins in the sampled population. Several factors have been found to influence the results of proteolysis and the subsequent analysis. Selecting an inappropriate denaturant for a protein mixture can result in poor digestion, and ultimately in low sequence coverage, lowering the confidence of protein identification. In this study, the effects of denaturant selection on digestion efficiency were examined using both a high-density immobilized Trypsin Spin Column and traditional solution digestion. The spin column format allowed complete protein digestion in 15 minutes without concomitant generation of tryptic autolytic fragments, resulting in greater sequence coverage for individual proteins even in a complex mixture.

A model system comprised of several proteins isolated from human serum was prepared and denatured in a variety of buffered systems. After reduction and alkylation, the protein solutions were digested utilizing the Trypsin Spin Column. Control digestions were performed overnight in solution with proteomics-grade trypsin. The digests were analyzed by SDS-PAGE and mass spectrometry to determine effectiveness of digestion and sequence coverage of the various proteins.

The Trypsin Spin Columns, coupled with optimal denaturant, provided extremely rapid and complete digestions, resulting in greater sequence coverage in a much shorter time than traditional solution digestion.

Introduction

In this study, the effects of denaturant selection on digestion efficiency were examined using both a high-density immobilized Trypsin Spin Column and traditional solution digestion. The spin column format allowed complete protein digestion in 15 minutes without generation of tryptic autolytic fragments (data not shown), resulting in high-confidence protein identification for individual proteins even in a complex mixture.

A protein solution was prepared and denatured in a variety of buffered systems. After reduction and alkylation, the protein solutions were digested utilizing the Trypsin Spin Column. For comparison, digestions were also performed overnight in solution with proteomics-grade trypsin. The digests were analyzed by SDS-PAGE and mass spectrometry to determine the effectiveness of digestion and sequence coverage of the various proteins.

The Trypsin Spin Columns, coupled with optimal denaturant, provided extremely rapid and complete digestions, resulting in greater sequence coverage in a much shorter time than traditional solution digestion. By immobilizing the trypsin at a high density on silica and using it in a spin column format, complete digestion can be achieved in 15 minutes or less.

Using a model system of proteins isolated from human serum, with optimal denaturation conditions and Trypsin Spin Columns, we observed more complete proteolytic digestion and achieved highly confident identification of most proteins tested.

Materials

All reagents were obtained from, or prepared at, Sigma-Aldrich.

- Trypsin Spin Columns, Proteomics Grade (**Cat. No.** TT0010)
- Trypsin, Proteomics Grade (**Cat. No.** T6567)
- ProteoPrep™ Reduction and Alkylation Kit (**Cat. No.** PROTRA)
- Model proteins: albumin, gelsolin, catalase, carbonic anhydrase I, plasminogen, and antithrombin III (**Cat. Nos.** A7223, G1538, C3556, C4396, P5661, and A2221, respectively)
- Protein Extraction Reagent Type 4, a mixture of urea, thiourea, Tris buffer, and C_7BzO detergent (**Cat. No.** C0356)
- EZBlue™ Gel Staining Reagent (**Cat. No.** G1041)
- ProteoSilver™ Silver Stain Kit (**Cat. No.** PROTSIL1)
- Supelco Discovery® HS C18 HPLC Column, 5-µm particle size, 15 cm × 2.1 mm (**Cat. No.** 568502-U)
- Thermo Finnigan LTQ Mass Spectrometer

Methods

A solution of six proteins from human serum was prepared in ammonium bicarbonate. The solution was divided into portions and denatured using various methods. The denaturants used were heat (55 °C for 30 minutes), 8 M urea, 6 M guanidine, and a detergent lysis reagent containing urea, thiourea, and C_7BzO (**C0356**). Samples denatured with 50% acetonitrile and trifluoroethane were discarded due to precipitation of the protein. The proteins were then reduced and alkylated with tributylphosphine and iodoacetamide.

Trypsin Spin Columns were prepared for use by washing successively with 2 M urea, 100 mM ammonium bicarbonate, and water.

The reduced and alkylated protein solutions were diluted with 3 volumes of 100 mM ammonium bicarbonate. Aliquots (100 µL) of each solution (approximately 100 µg total protein) were applied to Trypsin Spin Columns and incubated with the immobilized trypsin for 15 minutes. The peptides were eluted from the spin column with water.

Additional aliquots of the diluted proteins were digested in solution. Proteomics-grade trypsin was dissolved in 1 mM HCl and added at a ratio of approximately 1:25 (w/w) to the protein. The proteins were incubated at 37 °C for an overnight period.

The peptides from the overnight solution digestions and the spin column digestions were acidified with TFA and diluted to the same concentration prior to analysis.

Procedure

1. Denature protein solutions

2. Reduce and alkylate

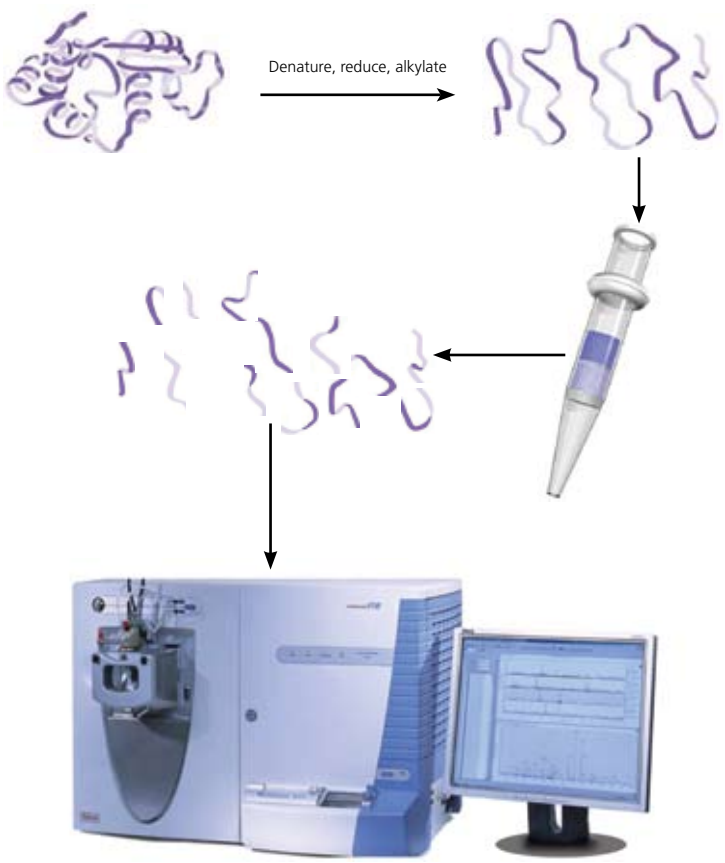
3. Wash spin column with urea, ammonium bicarbonate, and water

4. Apply denatured, reduced, and alkylated protein to spin column

5. Incubate at room temperature for 15 minutes

6. Elute peptides from spin column with water

7. Acidify and analyze



SDS-PAGE Results

To assess the thoroughness of proteolysis, the hydrolysis solutions were analyzed by SDS-PAGE and LC-MS/MS. Loss of intact protein and protein identification by peptide matches were used as measures of proteolytic efficiency.

Proteins denatured by four different methods were digested with either the Trypsin Spin Columns (15-minute incubation) or in solution (overnight incubation) and initially analyzed by SDS-PAGE with silver stain detection.

The gels were scrutinized for the presence of intact proteins, an indication of incomplete digestion. The proteins denatured with heat were poorly digested with both trypsin formats. The heat treatment caused precipitation and apparent aggregation of the proteins. The spin columns resulted in nearly complete digestion with C0356 and urea, although the C0356 gave the best results. Carbonic anhydrase, which can be stubborn to digestion by trypsin in solution, was well digested on the spin columns except with guanidine.

Trypsin Spin Columns Denaturant Comparison

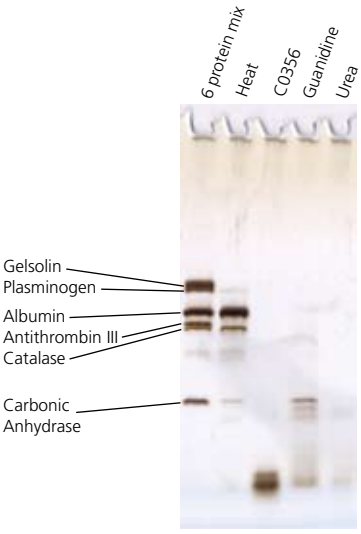


Figure 1: SDS-PAGE stained with ProteoSilver Silver Stain Kit illustrating digestion efficiencies with various denaturants.

Lane 1: Mixture of 6 proteins.

Lanes 2 through 5: The 6-protein mixture after denaturation, reduction, alkylation and tryptic digestion on Trypsin Spin Columns.

Heat was a very inefficient denaturant for this mixture. Albumin and catalase are left intact at high concentrations.

With guanidine, some intact carbonic anhydrase was detected.

The proteins were completely digested (no intact protein detected) with C0356 and urea.

Trypsin Spin Columns Speed of Digestion

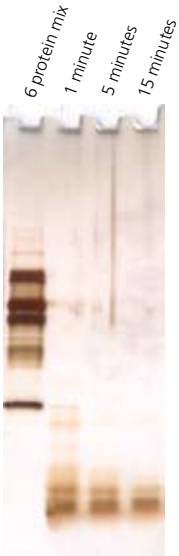


Figure 2: SDS-PAGE stained with ProteoSilver Silver Stain Kit.

The 6-protein mixture, reduced and alkylated in urea, was applied to Trypsin Spin Columns.

The peptides were eluted at three time points: 1, 5 and 15 minutes.

The majority of the proteins were completely digested after only 1 minute on the Trypsin Spin Columns. Some albumin and carbonic anhydrase were detected in the 1 minute sample, but at very low levels.

After 5 minutes, no intact protein was detected.

Lane 1: The initial protein mixture

Lane 2: After 1 minute digestion

Lane 3: After 5 minutes digestion

Lane 4: After 15 minutes digestion

LC-MS/MS Results

In the LC-MS/MS analysis, the peptides were separated on a Supelco HS C18 column with a gradient of water and acetonitrile incorporating 0.1% formic acid. Detection was performed with a Thermo Finnigan LTQ mass spectrometer using n^{th} order double play, monitoring the ten most intense ions. Dynamic exclusion was enabled. The data was analyzed by searching the UniProt human protein database with Bioworks/SEQUEST® software.

For each protein, the number of peptides associated with that protein, the XC score, and the sequence coverage were recorded. Results were compared for Trypsin Spin Column and solution digestion for each denaturant and for all denaturants with each digestion format.

Spin Column vs. Solution Digestion
Number of Peptides Identified when Denatured in Urea

Urea	Spin Column Digestion (15 minutes)			Solution Digestion (Overnight)		
	No. of Peptides	XC Score	Sequence Coverage	No. of Peptides	XC Score	Sequence Coverage
Gelsolin	7	17.4	17.3%	5	50.4	11.6%
Plasminogen	5	40.3	7.4%	5	30.3	5.3%
Albumin	50	210.3	30.4%	52	240.3	35.0%
Antithrombin III	13	82.3	32.1%	12	68.2	16.4%
Catalase	35	168.3	40.7%	27	150.3	36.6%
Carbonic Anhydrase I	22	110.3	58.1%	21	110.3	65.4%

Figure 3: Comparison of LS-MS/MS results from proteins denatured in urea then reduced, alkylated and tryptically digested on Trypsin Spin Columns (left) and in solution (right). For the majority of the proteins the sequence coverage following a 15 minute spin column digestion was the same or higher than that attained with overnight solution digestion.

Spin Column vs. Solution Digestion
Number of Peptides Identified and
Sequence Coverage Denaturant Comparison

Number of Peptides (Sequence Coverage)	Spin Column Digestion (15 minutes)			Solution Digestion (Overnight)		
Denaturant	**C0356**	**Guanidine**	**Urea**	**C0356**	**Guanidine**	**Urea**
Gelsolin	12 (22.6%)	9 (10.3%)	7 (17.3%)	13 (27.2%)	11 (14.3%)	5 (11.6%)
Plasminogen	8 (8.5%)	8 (5.8%)	5 (7.4%)	9 (7.4%)	6 (6.5%)	5 (5.3%)
Albumin	60 (35.5%)	56 (32.8%)	50 (30.4%)	59 (42.0%)	63 (36.0%)	52 (35.0%)
Antithrombin III	15 (28.9%)	4 (4.7%)	13 (32.1%)	17 (41.0%)	10 (14.0%)	12 (16.4%)
Catalase	32 (35.0%)	22 (36.1%)	35 (40.7%)	28 (37.3%)	22 (27.6%)	27 (38.6%)
Carbonic Anhydrase I	21 (61.5%)	7 (33.0%)	22 (58.1%)	21 (65.4%)	9 (38.9%)	21 (65.4%)

Figure 4: Comparison of LS-MS/MS results from proteins denatured in C0356, guanidine or urea, then reduced, alkylated and tryptically digested on Trypsin Spin Columns (left) and in solution (right).

The number of peptides identified and sequence coverage was similar for the two digestion formats, spin column and solution, even though the spin column digestion is complete after only 15 minutes and the solution digestion was allowed to incubate overnight.

The digestion was more complete for many of the proteins after denaturation in C0356 t

Conclusions

- For rapid digestion of proteins, Trypsin Spin Columns completely digested most proteins in 15 minutes or less when the appropriate denaturant was used.
- Trypsin Spin Column digestion often resulted in greater sequence coverage than overnight solution digestion.
- Using either the Trypsin Spin Column or traditional solution digestion the efficiency of the digestion was dependant on the selection of denaturant.
- All proteins tested digested completely, leaving no detectable intact protein on SDS-PAGE after silver staining, in C0356, a mixture of urea, thiourea and C_7BzO detergent using the Trypsin Spin Column.
- Heat denaturation, which is often used to avoid the addition of salts or detergents to a sample, resulted in poor digestions due to protein aggregation and precipitation.
- Mixed results were obtained using guanidine as the denaturant.