

# The Effect of Denaturant Selection on Tryptic Digestion: Comparing Rapid Proteolysis Using Trypsin Spin Columns to Traditional Solution Digestion

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## Overview

### Purpose

- To demonstrate the utility of the use of trypsin spin columns for rapid proteolysis.
- To compare and contrast the results of tryptic digestion of proteins using different denaturants.

### Methods

- A mixture of five proteins was prepared using four different denaturants. Each solution was reduced and alkylated.
- The reduced and alkylated proteins were tryptically digested in less than 15 minutes using trypsin spin columns or overnight in solution with Proteomics Grade Trypsin.
- The digests were analyzed by SDS-PAGE, HPLC, and MALDI-TOF-MS.

### Results

- The denaturant selection affected the degree to which each protein in the mixture was digested.
- The spin column and solution digestions produced differing results.
- Spin column digestion resulted in better sequence coverage by MALDI-TOF-MS.
- Most proteins are completely digested on the trypsin spin columns in 15 minutes.

## Introduction

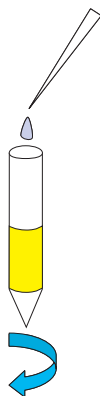
Tryptic digestion of proteins continues to be a workhorse method in proteomics. The peptides generated are analyzed by mass spectrometry and used to identify and characterize the sampled protein population. This important technique can require several hours, if not overnight, to complete. Increased digestion speed for the generation of same-day data is highly desirable. Several factors influence the results of proteolysis, including time, temperature, denaturant, protease concentration, and buffer. In this study, the effects of denaturant selection and contact time on digestion efficiency are examined using a high-density immobilized trypsin mini spin column and traditional solution digestion. The newly developed spin column format allows complete digestion in 15 minutes without the generation of significant tryptic autolytic fragments.

## Materials

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

- Trypsin Spin Columns, Proteomics Grade (Product Code H1914)
- Trypsin, Proteomics Grade (Product Code T6567)
- ProteoPrep™ Reduction and Alkylation Kit (Product Code PROT-RA)
- Model proteins:  $\beta$ -galactosidase, bovine serum albumin, fumarase, carbonic anhydrase, lysozyme and insulin oxidized B chain (Product Codes G5635, A3059, F1757, C4396, L6876 and I6383)
- Protein Extraction Reagent Type 4, a mixture of urea, thiourea, Tris buffer and C<sub>12</sub>BzO detergent (Product Code C0356)
- EZBlue™ Gel Staining Reagent (Product Code G1041)
- ProteoSilver™ Silver Stain Kit (Product Code PROT-SIL1)
- Supelco Discovery® C18 HPLC Column, 15 cm × 4.6 mm, 5  $\mu$ m (Product Code 504955)
- ProteoMass™ Peptide and Protein MALDI-MS Calibration Kit (Product Code MSCAL1)

## Process Overview



**Figure 1: The use of the trypsin spin column.**

Wash the spin column with buffer using centrifugation. Apply the protein sample to the column. Centrifuge to pull the sample into the resin bed. Incubate at room temperature for 15 minutes. Elute the peptides from the column with water or buffer. Analyze by SDS-PAGE, LC-MS, or MALDI-TOF-MS.

## Methods

A model system of mixed purified proteins and proteins extracted from HeLa cells were subjected to proteolysis using either traditional in-solution trypsin digestion or rapid digestion utilizing the trypsin spin column. Protein solutions were prepared in a variety of denaturants and reduced and alkylated prior to dilution in digestion buffer. The peptides generated were analyzed by SDS-PAGE, HPLC, LC-MS, or MALDI-TOF-MS.

A solution of  $\beta$ -galactosidase, bovine serum albumin (BSA), fumarase, carbonic anhydrase, and lysozyme was prepared in water. The protein mixture was diluted into 30% acetonitrile (ACN) in ammonium bicarbonate, 30% ACN in Tris buffer, C0356 (Protein Extraction Reagent Type 4, a chaotropic reagent containing urea, thiourea and C<sub>12</sub>BzO detergent), 8 M guanidine, pH 8, or 8 M urea to denature. The proteins were then reduced with tributylphosphine and alkylated with iodoacetamide.

HeLa cells, which were lysed with RIPA buffer, were diluted with C0356 prior to reduction and alkylation.

The trypsin spin columns were prepared for use by washing with 2 M urea, then equilibrated in reaction buffer, either 100 mM ammonium bicarbonate or 25 mM Tris pH 8. The reduced and alkylated proteins were diluted with 3 volumes of buffer and 100  $\mu$ L of sample, containing approximately 30  $\mu$ g of total protein, applied to the column. Following incubation for up to 15 minutes at room temperature, the resultant peptides were eluted with 150  $\mu$ L water and analyzed.

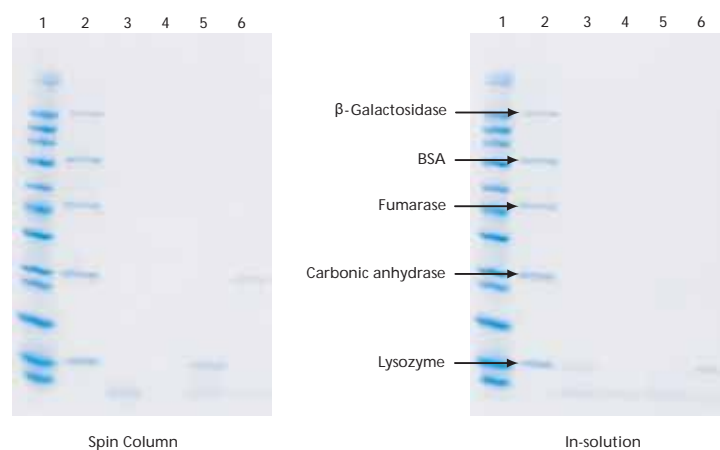
Solutions for traditional tryptic digestion in solution were prepared in an analogous manner. Following reduction and alkylation, the proteins were diluted with 3 volumes of ammonium bicarbonate. Proteomics Grade Trypsin, reconstituted in 1 mM HCl, was added at an approximate ratio of 1:40 ( $\mu$ g trypsin to  $\mu$ g protein) and incubated at 37 °C overnight.

The digests of the model protein mixture were analyzed by SDS-PAGE and MALDI-TOF-MS to demonstrate digestion efficiency and sequence coverage. The HeLa cell digests were analyzed by SDS-PAGE.

To demonstrate cleavage accuracy, a peptide with two trypsin cleavage sites, insulin oxidized B chain, was digested and analyzed by HPLC. The eluted peaks were identified by LC-MS (data not shown). The fragments generated with the trypsin spin column in 1, 5 and 15 minute incubations was compared to those from in-solution digestion at the same time points and after 1 hour.

## Results

### Digestion of a Model Mixture of Purified Proteins



**Figure 2. Coomassie stained SDS-PAGE gel of digested model proteins.**

Tryptic digestion of samples denatured in acetonitrile, C0356, guanidine and urea in ammonium bicarbonate was evaluated using both trypsin spin column and solution digestion. On the left panel, the products from overnight trypsin spin column digestions are shown. On the right panel, the products from 15 minute trypsin spin column digestions are shown. Lane 1: M4038, Wide Range Molecular Weight Markers. Lane 2: The original five protein mixture. Protein mixtures denatured, reduced, and alkylated and digested in: Lane 3: ACN. Lane 4: C0356. Lane 5: Guanidine. Lane 6: Urea. The digestion buffer is 100 mM ammonium bicarbonate. The same experiment was conducted with 25 mM Tris, pH 8 with similar results (data not shown).

Proteins denatured in C0356 (Lane 4 on each gel) are completely digested in both systems. Lysozyme digested well in guanidine on the spin column, but not in solution. Carbonic anhydrase digested well in urea in solution, but not on the spin column. Overall digestion efficiency is similar for 15 minute spin column digestion and overnight solution digestion.

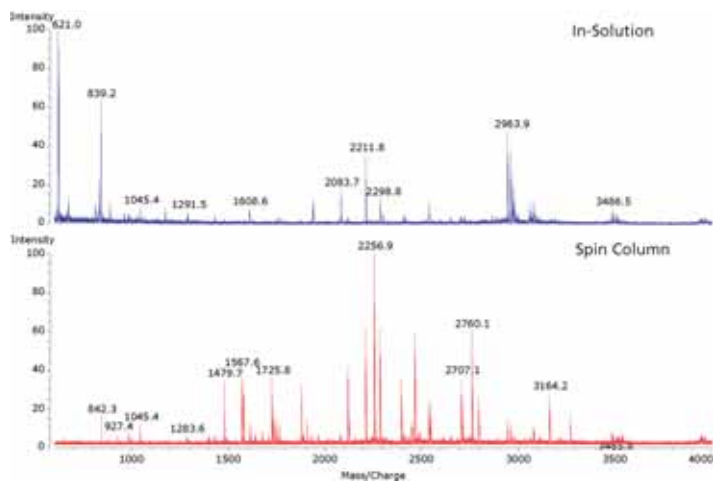
## Digestion of a Complex Protein Solution



**Figure 3. Silver stained SDS-PAGE gel of tryptic digestion of HeLa cell extract.** Lanes 1 and 9: HeLa cells lysed in RIPA buffer were diluted into C0356 and reduced and alkylated. Lanes 2, 3 and 4: Time course digestion on trypsin spin columns. 1, 5 and 15 minutes, respectively. Lanes 5, 6, 7 and 8: Time course digestion in solution. 1, 5 and 15 minutes and overnight at 37 °C, respectively.

In 1 minute on the spin column, the HeLa cell proteins are more completely digested than after overnight in solution.

## Sequence Coverage and Number of Peptides Identified from Digestion of the Five-Protein Mixture by MALDI-TOF-MS

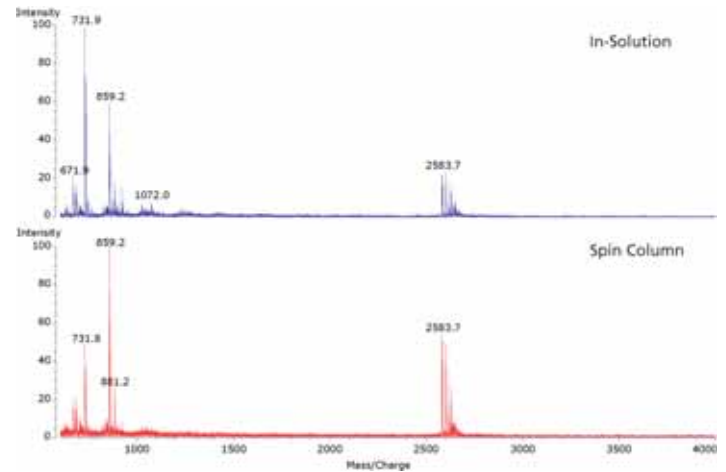


	In-Solution	Spin Column
β-Galactosidase	13% 8 peptides	10% 6 peptides
BSA	48% 15 peptides	46% 21 peptides
Fumarase	8% 2 peptides	56% 18 peptides
Carbonic Anhydrase	18% 3 peptides	34% 5 peptides
Lysozyme	68% 6 peptides	44% 4 peptides

**Figure 4. Sequence coverage and number of peptides identified by MALDI-TOF-MS.** The five-protein mixture digests were analyzed by MALDI-TOF-MS and the sequence coverage of each protein calculated. In 4 out of 5 cases, the sequence coverage obtained with the spin column digest was similar to or better than that obtained from the solution digest. The proteins were reduced, alkylated, and digested in urea.

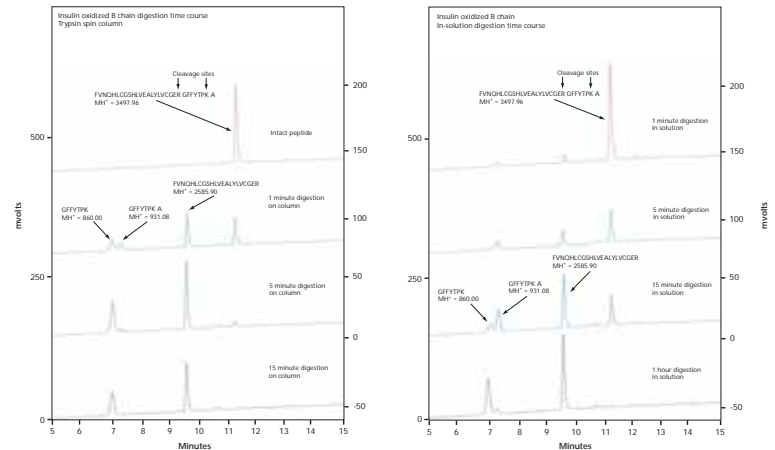
The sequence coverage for fumarase and carbonic anhydrase was significantly enhanced with the spin column digestion.

## Cleavage Accuracy



**Figure 5: MALDI-TOF-MS of digested insulin oxidized B chain.** Insulin oxidized B chain was denatured in urea and digested on trypsin spin columns and in solution. The digests were analyzed by MALDI-TOF-MS and HPLC to verify that the expected peptides are obtained and that the immobilized trypsin cleaves at the expected residues, in a short time, without missed cleavages. Top panel: Solution digest. Bottom panel: Spin column digest.

The spin column and solution digestion give nearly identical results by MALDI-TOF-MS.



**Figure 6: Digestion time course of insulin oxidized B chain, monitored by HPLC.** The left panel illustrates the rapid, accurate cleavage of insulin oxidized B chain using the trypsin spin columns. When compared to the right panel, the in-solution digest analyses, the 5 minute spin column digest gives similar results to the 1 hour solution digest.

The digestion of insulin oxidized B chain is faster and more accurate with the trypsin spin columns than with solution digestion.

## Conclusions

- For rapid digestion of proteins, trypsin spin columns will completely digest all proteins in 15 minutes or less when the appropriate denaturant is used.
- Using the spin column or traditional solution digestion, the efficiency of the digestion is dependent on the selection of the denaturant.
- All of the proteins tested digested completely, leaving no detectable intact protein on an SDS-PAGE gel, in C0356, a mixture of urea, thiourea and C<sub>7</sub>BzO detergent. Acetonitrile was also an effective denaturant for most of the proteins.
- Mixed results are obtained using guanidine or urea as the denaturant.
- Enhanced MALDI-TOF-MS sequence coverage data was obtained using the trypsin spin columns for digestion.