New LC-MS Workflows Enabled by Efficient In-Line Digestion

P1825

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Introduction

The objective of bioanalytical workflows is generally to detect, identify, and sometimes quantify proteins. Despite significant advances in instrument capabilities, the analysis and detection of intact proteins remains a challenging task. Consequently, the majority of workflows center on the analysis of peptides, either proteomics or peptide mapping of biopharmaceuticals.

Trypsin is a commonly used protease that cleaves at the carboxyl side of the amino acids lysine and arginine; the generated peptides are easily separated and detected by LC-MS techniques and the predictable cleavage sites can facilitate data analysis. Usually the digestion is performed off-line and in solution, which is a time consuming and laborious process. Immobilizing the protease on a monolithic column creates several advantages: It enables an automated, in-line process, accelerates the digestion, and opens up new LC-MS workflows, factors that are relevant to all fields that face the challenges of intact protein analysis. The use of a monolithic carrier for immobilization provides open flow-through channels for fast, efficient digestion.

Tryptic Digestion

Trypsin is a serine protease produced by the pancreas of many vertebrates to hydrolyze proteins. It cleaves peptide chains mainly at the carboxyl side of the amino acids lysine (K) and arginine (R) unless they are followed by proline (P).

This specificity makes trypsin a popular choice for protein identification. Identification of sequence is the first step in proteome analysis. The conventional method of in-solution digestion using sequence specific proteases is time consuming due to slow kinetics and subject to contamination from external sources, making it unreliable.

Typical digestion protocols require from 1:100 to 1:20 (w/w) trypsin to protein ratios, with digestion times ranging from 1 hour to overnight. Upon analysis, if a significant amount of trypsin is used, peaks will be observed at 842 and 2211 *m/z* on the mass spectrometer. These peaks are the result of autocatalytic trypsin digestion and cleavage at arginine residues, which are not protected by reductive methylation.

Immobilized Tryptic Digestion

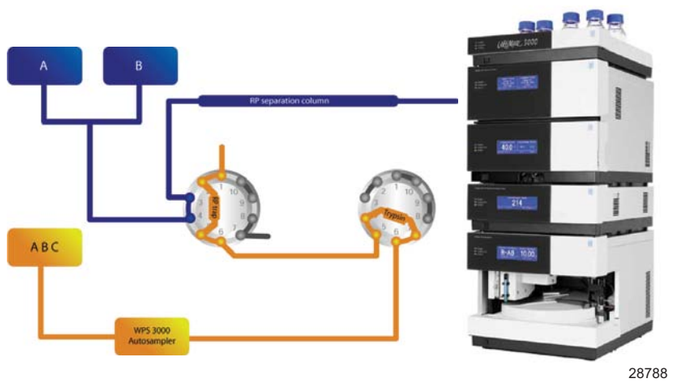
Immobilizing enzymes on the surface of a substrate provides significantly improved catalytic efficiency. Because digestion rate is proportional to enzyme concentration, increasing the trypsin to protein ratio will accelerate the rate at which digestion occurs; however, in solution this also increases the amount of auto-digestion. Immobilizing the enzyme on a surface allows an increase in local enzyme concentration while maintaining sufficient distance between the trypsin units, to prevent auto-digestion. Also, when protein concentration is unknown, in-line digestion offers a benefit since the protein to trypsin ratio does not have to be adjusted.

This digestion technique employs linear flow through without any retention mechanism, the mass of protein digested can be increased by increasing the sample volume injected and passed through the digestion column. The digestion products are then simply preconcentrated onto the trap column. This allows fast digestion in minutes rather than hours, and a maximum digestion capacity that is determined by the trap column loadability.

A Complete Solution

The immobilized trypsin columns have been designed to allow integration into existing Thermo Scientific Dionex UltiMate™ 3000 RSLCnano system workflows. Figure 1 shows the standard preconcentation nano LC configuration, expanded with an online digestion column. Mounting the column on the valve allows placement of the column in-line or out-line depending on the experiment. The preconcentration column is perfectly suited for washing off the digestion buffer prior to MS analysis.

FIGURE 1. System schematic for in-line protein digestion with reversed-phase separation.



In the in-line configuration, the protein is prepared in digestion buffer and loaded onto the digester using the loading pump. As the protein passes through the digester, it is cleaved by the immobilized enzyme. The resulting peptides are trapped on the trap column and desalted. Separation and detection takes place as in any preconcentration experiment by placing the trap in-line with the separation column and using a nano LC gradient for the elution. Figure 2 shows a comparison of in-solution and in-line cytochrome C digestion.

FIGURE 2. Comparison of in-solution cytochrome C digest (Dionex P/N 161089) and in-line digestion of cytochrome.

Experimental

Digestion Column: 0.32 × 200 mm monolith prototype

Option 1 for higher mass loading

Trap Column: Thermo Scientific Acclaim™ PepMap™100, C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, (P/N 160454)

Analytical Column: Acclaim PepMap RSLC C18, 2 µm, 100 Å, 300 µm i.d. × 150 mm (P/N164537)

Option 2 for smaller samples

Trap Column: Acclaim PepMap100, C18, 5 µm, 100 Å, 100 µm i.d. × 2 mm, (P/N 164564)

Analytical Column: Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. × 150 mm (P/N 164568)

Loading Eluent: 2% acetonitrile in water, plus 0.05% trifluoroacetic acid

Digestion Solution: 50 mM ammonium bicarbonate, pH 8

Gradient Eluent A: Water, 0.05% trifluoroacetic acid

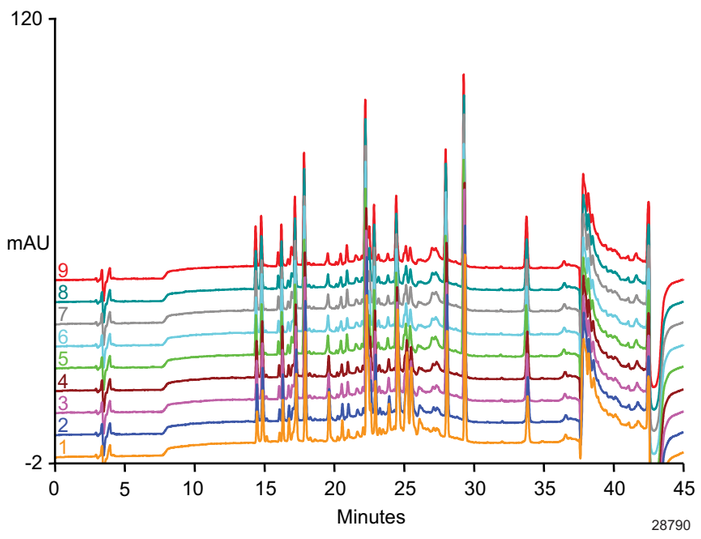
Gradient Eluent B: 80% acetonitrile, 20% water, 0.04% trifluoroacetic acid

Loading/Digestion Flow Rate: 2 µL/min unless otherwise noted

Digestion Conditions and Trypsin Stability

Trypsin is most active at ~pH 8 and 37 °C, and is reversibly deactivated at pH <4. In order to reduce equilibration times, digestion is carried out by preparing the sample in digestion buffer so that the sample environment is optimal for digestion. The sample is carried though the digester using eluent optimal for desalting the trap column. The trypsin is inactive in the loading buffer (~pH 3) and is rapidly reactivated upon contact with the sample in digestion buffer (pH 8). The surface-immobilized trypsin is stable to continuous contact with acetonitrile and TFA. Figure 3 shows digestion repeatability as well as reproducible analysis results.

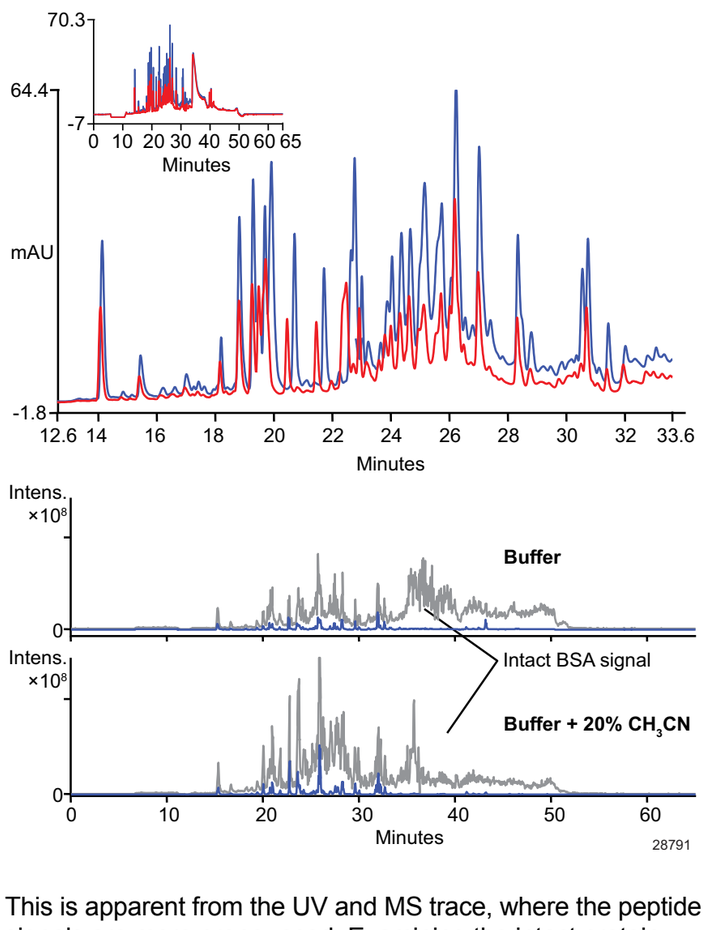
FIGURE 3. Repeat digestions of cytochrome C separated using reversed phase.



Influence of Protein Structure on Digestion Efficiency

Cytochrome C is known to be easily digested. However, with many proteins, secondary structure prevents complete digestion without pretreatment of the sample. Figure 4 shows the digestion of BSA when diluted using only buffer and using buffer with 20% acetonitrile to denature the protein. Unfolding allows enzyme access to the internal protein structure to enable more complete digestion.

FIGURE 4. In-line digestion of bovine serum albumin with (blue) and without (red) the use of solvent. The gray trace represents the TIC



This is apparent from the UV and MS trace, where the peptide signals are more pronounced. Examining the intact protein peak in the MS TIC, this difference is clear.

In addition to the sample solvent, the amount of sample and the digestion time (influenced by flow rate) were changed. Table 1 and Figure 5 show the results for BSA. The sequence coverage does not significantly increase when adding acetonitrile, which is explained by a more complete conversion from the improved unfolding, but not necessarily different peptides. The shorter digestion times (4 µL/min) typically resulted in poorer performance of the conversion.

FIGURE 5. Sequence coverage of BSA digest with and without solvent present.

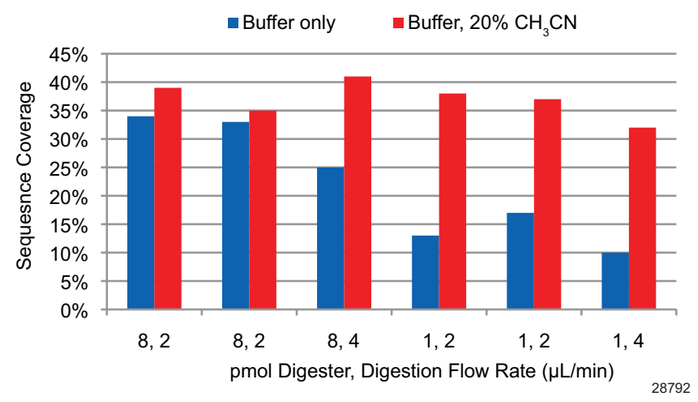


Table 1. Comparison of the Effect of Different Digestion Conditions on the Sequence Coverage and Mascot Score of BSA

Sample Solvent	Amount (pmol)	Digestion Flow (µL/min)	Seq Cov	Mascot Score
Buffer	8	2	34%	1262
	8	2	33%	1229
	8	4	25%	1082
	1	2	13%	571
	1	2	17%	601
	1	4	10%	341
Buffer + 20% CH_3CN	8	2	39%	1314
	8	2	35%	1251
	8	4	41%	1378
	1	2	38%	1364
	1	2	37%	1277
	1	4	32%	1233

The limited effect of adding acetonitrile is explained by the protein sequence. BSA has a total of 35 cysteine residues; in the detected peptides, only 4 of the 35 are present. The remaining cysteine residues create an internal structure that prevents efficient digestion. A stronger denaturing step (reduction and alkylation) than adding acetonitrile is required.

Cysteines: 35 total, 4 detected, 31 not detected.

1 MKWVTFISLL LLFSSAYSRG VFRR**DTHKSE** **IAHRFKDLGE** **EHFKGLVLIA**
51 **FSQYLQQCPF** **DEHVKLVNEL** **TEFAK**TCVAD ESHAGCEKSL HTLFGDELCK
101 VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
151 KADEK**KFWGK** **YLYEIARRHP** **YFYAPELLYY** **ANK**YNGVFQE CCQAEDKGAC
201 LLPK**IETMRE** KVLASSARQR LRCASIQKFG ERALKAWSVA R**LSQKFPKAE**
251 **FVEVTKLVTD** **LTK**VHKECCH GDLLECADDR ADLAK**YICDN** **QDTISSK**LKE
301 CCDKPLLEK**S** **HCIAEVEK**DA IPENLPPLTA DFAEDKDVCK NYQEAK**DAFL**
351 **GSFLYEYSRR** **HPEYAVSVLL** **R**LAKEYEATL EECCAKDDPH ACYSTVFDKL
401 K**HLVDEPQNL** **IK**QNCDQFEK **LGEYGFQNAL** **IVR**YTR**KVPQ** **VSTPTLVEVS**
451 **R**SLGKVGTRC CTKPESERMP CTEDYLSLIL NR**LCVLHEK**T PVSEKVTKCC
501 TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIK**KQT**
551 **ALVELLK**HKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPK**LVV**
601 **STQTALA**

A mixture of three proteins (carbonic anhydrase, ribonuclease A, and myoglobin) was injected onto the digestion column (Figure 6.) The amino acid composition reveals why ribonuclease A is not identified; it is the only protein with cysteine residues and is therefore tightly folded. The other two proteins were identified, with sufficient sequence coverage for identification.

FIGURE 6. Mixture of three proteins injected onto the digestion column.

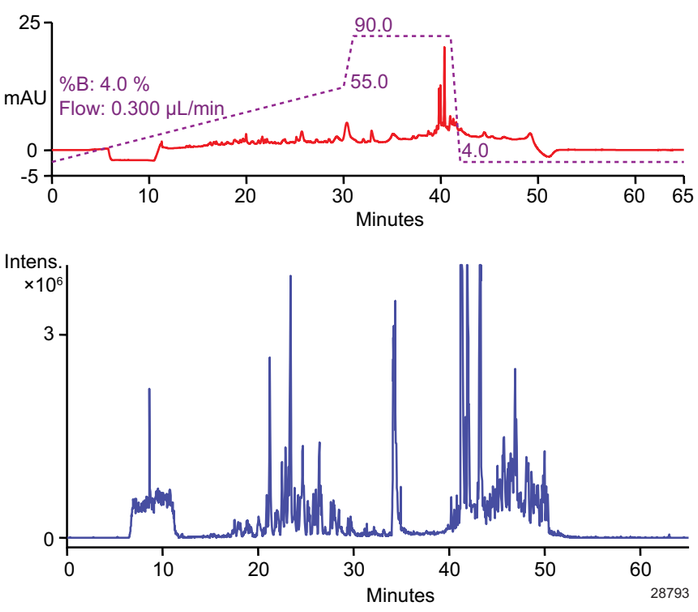


Table 2. Amino Acid Composition of Proteins Used in Figure 6

Amino Acid Composition	Myoglobin	Ribonuclease A	Carbonic Anhydrase
Cysteine	0	8	0

Conclusions

- In-line digestion works and is aided by dissolving the proteins in a denaturing solvent.
- The cysteine sulfur bridges are not broken by the addition of acetonitrile and this is the reason for the absence of digestion (ribonuclease A) or lower sequence coverage (BSA)
- The robustness of the digestion columns is excellent: repeated cycles of trifluoroacetic acid and acetonitrile in the solvents did not influence the digestion performance.
- The digestion column is easily integrated in existing RSLCnano workflows.

References

1. Polgár, L. *Cell. Mol. Life Sci.* **2005,** *62,* 2161–72.

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