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Miniaturisation of Trypsin Immobilised Enzymatic Reactor for Protein Digestion

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Abstract

Pre-activated CIMmic™ monolithic columns with 100 µL bed volume were immobilized with trypsin from bovine pancreas. This small format allows coupling to HPLC for on-line protein digestion and the syringe (manual) operation of the IMER. Pre-treated samples (denatured, alkylated, and ultra-filtered) are injected into the column, and the eluate (tryptic digests) are subjected to LC-ESI-MS-MS analysis for protein identification and post-translational modification (PTM) determination.

Introduction

Miniaturized immobilized enzymatic reactors can be used for small-scale digestion of proteins. There is a need for such devices; small-scale devices are used to process analytical sample quantities or as proof of concept before protein digestion at a larger scale (see references). This application note compares the performance of a flow-through miniaturized immobilized enzymatic reactor (µIMER) with in-solution batch digestion of simple proteins and complex matrices. Automation of peptide analysis by coupled LC-MS is explored as an option to increase throughput. In the cases evaluated, the miniaturized immobilized enzymatic reactor offered comparative results to overnight in-solution digestion within less than 10 minutes.

Materials

- Digestion column: CIMmic™ ALD (aldehyde) immobilized with trypsin from bovine pancreas
- HPLC system: PATfix® system or equivalent

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Methods

Operating conditions for µIMER digestion

- Method: Allow the column to equilibrate to working temperature for 20-30 min before the first injection. Equilibrate the µIMER with at least 10 column volumes (CV) of digestion buffer
- Digestion buffer: 20 mM ammonium bicarbonate pH 8.0 | Flow rate: 0.5 mL/min | Detection: 230 nm UV absorbance | Column temperature: 37 °C | Injection volume: 50 µL (at concentration 0.26 mg/mL)

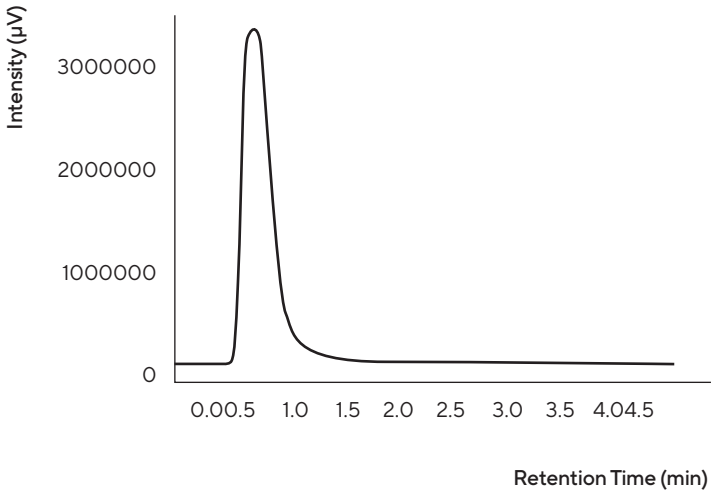

Figure 1: Representative chromatogram of tryptic digest eluting from the miniaturised immobilised enzymatic reactor.

Results

Digestion of simple proteins

Human serum albumin (HSA) is the most abundant plasma protein. HSA microheterogeneity has recently attracted the scientific community's interest because of its important implications both in clinical and pharmacological fields. The protein was chosen as a model to evaluate µIMER performance.

A 0.5 mL aliquot of denatured and iodoacetamide treated HSA (1.0 mg/mL) was ultra-filtered using AmiconUltra-0.5 mL, 10 kDa, Centrifugal Filters (Millipore) to remove reagents and injected on the HPLC system. The flow through fraction was collected for 2 minutes. A control sample consisting of HSA 0.26 mg/mL (50 µL) was digested in batch overnight with trypsin from bovine pancreas (Sigma Aldrich). HSA/trypsin ratio (w/w) was 100. For LC-ESI-MSMS analysis, the collected fraction was adjusted to a final volume of 500 µL. An aliquot of 100 µL was then subjected to LC-ESI-MSMS analysis.

The use of µIMER allowed the complete digestion of HSA; no undigested protein was detected in the collected fraction. Identification of HSA based on data bank search resulted in excellent scores and high match values, which were even more significant than those achieved with classical overnight digestion (see table below). Moreover, the proposed workflow granted a protein coverage higher than 80%.

Several post-translational modifications (PMTs) affecting protein structure were also identified, including glycation (Lys195; Lys233; Lys281; Lys525), Cys sulfinylation (Cys34), Cys sulfonylation (Cys34) and methionine oxidation (Met87; Met298; Met329; Met446).

Digestion of HMW human plasma protein fraction

To evaluate the performance of the µIMER for digestion of complex mixtures of proteins, a high molecular weight (HMW) protein fraction of human plasma was used.

The high molecular weight (HMW) protein fraction was isolated by ultrafiltration using AmiconUltra-0.5 mL, 100 KDa, Centrifugal Filters (Millipore). The fraction was reduced with DTT, alkylated by iodoacetamide, and ultra-filtered before injection. The flow-through fraction was collected for 4 minutes. For comparison, a 50 µL-aliquot of the HMW plasma protein fraction was diluted to 0.26 mg/mL with ammonium bicarbonate 20 mM pH 8 and digested in batch overnight using trypsin from bovine pancreas (Sigma Aldrich). HMW protein fraction/trypsin ratio (w/w) was 100. For LC-ESI-MS-MS analysis, the fraction collected within 4 minutes was dried and re-suspended with 60 µL of water. Aliquots of 50 µL were subjected to LC-ESI-MS-MS analysis.

The number of peptides generated by CIMmic™ trypsin µIMER was 1071 ± 29, while the number of the identified proteins after databank search was 265 ± 41 (See Figure above). These results are only slightly lower than those obtained by the overnight in batch digestion (number of peptides: 1191 ± 1; the number of identified proteins: 273 ± 18). Considering the high number of proteins, the result can be regarded as satisfactory (3% difference in identified plasma protein).

	CIMmic™ µIMER	In batch
Score	2263 ± 12	1247 ± 178
Matches	72 ± 5	53 ± 0
Coverage	83% ± 1%	67% ± 3%

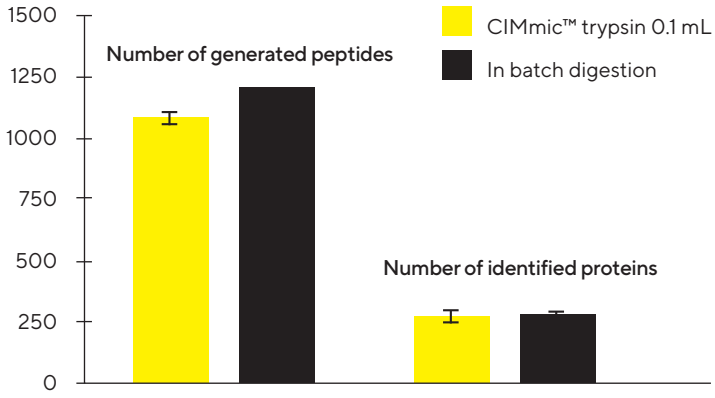

Figure 2: Peptide count and proteins identified during on-line digestion in HMW fraction of human plasma using µIMER and batch digestion in-solution.

Conclusion

Miniaturised immobilized enzymatic reactors provide an improved platform for protein digestion. The chromatographic fraction is collected within minutes and contains the mixture of peptides resulting from the injected sample's on-line digestion. CIMmic™ trypsin µIMER offered similar or better performance than classical in-solution digestion, with evident advantages related to time-saving and higher automation degree thanks to its insertion into an HPLC system direct hyphenation with an LC-ESI-MS-MS system for the online digest analysis. The µIMER can be regenerated within few minutes and used for subsequent analysis. Furthermore, the use of immobilized trypsin prevents trypsin autodigestion, eliminating interferences.

BIA Separations offers CIMac™ Trypsin analytical column (0.1 mL bed volume), pre-immobilized with trypsin. Besides, activated monolith chemistries, available in CIMmic™ (0.1 mL bed volume) and CIMmultus™ (1 mL and larger) formats, provide a platform for immobilization of custom enzymes or proteins for affinity chromatography. Combined, CIMmic™ and CIMmultus™ offer a solution to evaluate ligand immobilization at a small scale (simplified, manual operation) and then increase the column's size to process larger sample quantities.

References

Naldi M, Černigoj U, Štrancar A, Bartolini M. Towards automation in protein digestion: Development of a monolithic trypsin immobilized reactor for highly efficient on-line digestion and analysis. Talanta, 2017, 167, 143-157.

Chen, W. Q., Obermayr, P., Černigoj, U., Vidič, J., Panić-Janković, T., & Mitulović, G. (2017). Immobilized monolithic enzymatic reactor and its application for analysis of in-vitro fertilization media samples. Electrophoresis, 38(22-23), 2957-2964.

AN058: Applicability of CIM®-monolith based immobilized trypsin reactors for continuous β-Lactoglobulin hydrolysis.

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