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LC-MS and MALDI-MS Investigation of Tryptic Protein Digestion: Towards Protein Digestion Standards

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Overview

- Proteolysis is thought to be a well understood process, however, many exceptions to the basic cleavage rule (cleave after K or R, but not when followed by P) have been reported (**Table 1**).
- In this study the selected proteins were digested and analyzed by LC-MS, LC-MS/MS, and MALDI-MS in order to evaluate the fidelity of trypsin (**Figures 1-4**).
- The examples demonstrating the variability of proteolytic cleavage are given (**Figure 1**). This variability makes it difficult to use protein digests as HPLC and MS standards and potentially complicates the proteome analysis of global protein digests.
- Ionic surfactant (RapiGest™ SF) was found to enhance the speed and completeness of the digestion (**Figure 2**) (Yu et al. *Anal Chem*. 2003 Nov 1, 75(21): 6023-8, Yu et al. *Rapid Commun Mass Spectrom*. 2004; 18(6): 711-5, Suder et al. *Rapid Commun Mass Spectrom*. 2004; 18(7): 822-4.)
- A set of standards was prepared by digesting selected proteins with trypsin using RapiGest™ SF for improved tryptic digestion.
- RapiGest™ SF decomposes to MS (LC/MS and MALDI-TOF) non-interfering by-products upon acidification
- Digestion using RapiGest™ SF results in fewer incompletely cleaved tryptic peptides. However, certain motifs in the primary amino acid sequence are still resistant to cleavage (**Figure 3**). Some of those motifs differ from those reported in the literature (**Table 1**).
- Standards were prepared to be free of salts, undigested protein, trypsin, and alkylation and reduction agents.
- Standards are useful for MS/HPLC instrument testing, calibration, MALDI-MS peptide mass fingerprinting and 2D-HPLC method development.

Experimental

Yeast alcohol dehydrogenase bovine serum albumin, bovine hemoglobin, and rabbit phosphorylase b were reduced and alkylated (DTT, iodoacetamide, Sigma) prior to digestion (sequencing grade trypsin, Promega), yeast enolase and cytochrome c were digested without reduction/alkylation. The acid-labile surfactant RapiGest™ SF was added to the digestion buffer (50 mM ammonium bicarbonate, pH 7.9). Progress of the digestion was monitored by LC-MS. The complete digestion of protein was achieved only in the presence of surfactant. Digested proteins were separated using BioSuite™ C_{18} 3 µm PA-A, 150 x 2.1 mm columns with on-line MS detection (Waters Micromass® ZQ™ 4000 single quadrupole instrument). Mobile phases were A: 0.02% TFA in water and B: 0.016% TFA in acetonitrile. Gradient conditions were 0.8% B/minute with a flow rate of 0.2 ml/min. Samples were also analyzed by MALDI-MS using alpha-cyano-4-hydroxy-cinnamic acid as the matrix

HPLC System: Waters Alliance® 2795 Separations Module (Milford, MA) with a 996 PDA detector and online MS detection using a Waters Micromass® ZQ™ 4000

MALDI TOF MS: Waters MALDI® *LR* TOF MS, Reflectron mode

Figure 1: LC-MS analysis of cytochrome c tryptic digest. A—incomplete digestion, B–advanced digestion (extended digestion time and/or addition of trypsin). Some peptides partially resistant to cleavage (T9-10, T12-13, and T13-14) were digested in case B, however, the T4-5 peptide was highly resistant to tryptic cleavage.

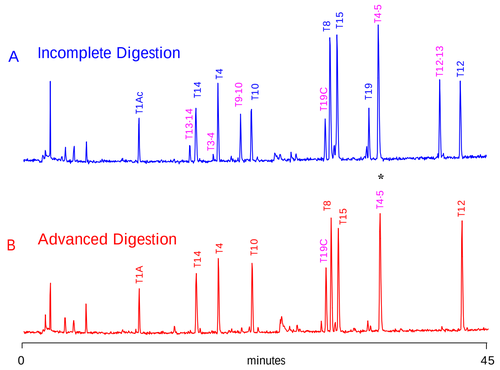


Figure 2: Comparison of an enolase digest with and without RapiGest™ SF. Enolase was digested with sequencing grade trypsin at a ratio or 50:1 (w/w) protein to trypsin with and without 0.05% RapiGest™ SF present in the sample. It is clear that enolase is more completely digested in the presence of RapiGest™ SF.

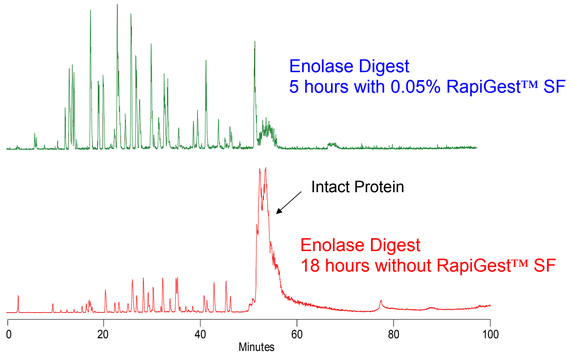


Figure 3: LC-MS analysis of MassPREP™ protein digestion standards. Number of tryptic peptides detected and identified by their molecular weight is indicated. A complete and reproducible digestion of proteins was achieved with the aid of RapiGest™ SF acid labile surfactant. The most resistant missed cleavages are indicated with an asterisk.

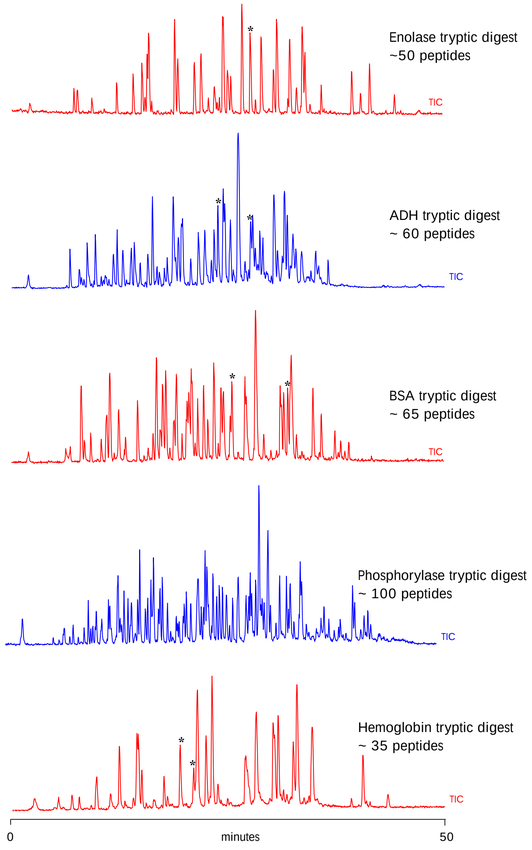
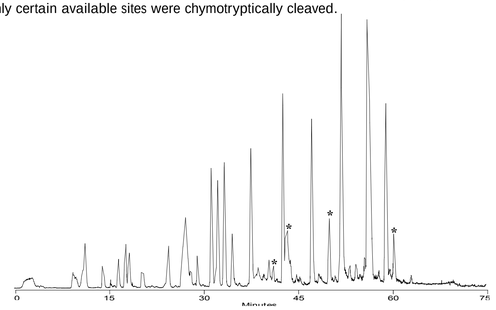


Table 1: Motifs that are partially resistant to trypsin cleavage. The column on the left lists the partially resistant motifs that are listed in the Swiss-Prot database. The column on the right lists the resistant motifs identified in this study (Figure 1 and Figure 3 peaks labeled with an asterisk). Unique motifs are listed in red.

Swiss-Prot Partially Resistant Motifs	Resistant Motifs Found in this Study
CKY	EKD
DKD	GKE
CKH	RKV
CKD	DKD
KKR	DKL
RRH	DKG
RRR	VKV
CRK	QKC
DRD	
RRF	
KRR	

Figure 4: LS-MS/MS analysis of MassPREP™ enolase digestion standard. Peaks labeled with an asterisk result from secondary chymotryptic activity of Promega trypsin. Interestingly, only certain available sites were chymotryptically cleaved.



Conclusions

- The LC-MS monitoring of protein tryptic digestion suggests that proteolysis rarely proceeds to absolute completion. Therefore, it is difficult to reproducibly prepare protein digestion standards.
- The use of RapiGest™ SF acid–labile surfactant as an additive to the digestion buffer increased the speed and completeness of the digest.
- RapiGest™ SF improves digestion by denaturing/unfolding the proteins, making them more amenable to proteolysis and generating fewer incompletely cleaved peptides.
- The surfactant had a positive impact on the completeness of the digestion, however, highly resistant motifs were not cleaved.
- Several new resistant motifs were found in this work (Table 1).
- Our results suggest that RapiGest™ SF is useful for preparing more complete and more reproducible global protein digests.
- The five protein digests were prepared and qualified by LC-MS and MALDI-MS. They were found to be useful as samples for 2D-HPLC method development.