IMPROVED BIOPHARMACEUTICAL PEPTIDE MAPPING WORKFLOWS USING A NOVEL AUTOLYSIS-RESISTANT TRYPSIN ENZYME

Samantha Ippoliti[1], Nick Zampa[1], Ying Qing Yu[1], Matthew A Lauber[1], Leslie Napoletano[1], Scott Berger[1]
Waters Corporation, Milford, MA

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INTRODUCTION

Peptide mapping provides a wealth of information about a protein therapeutic. Through digestion of protein chains down to peptide level, it is possible to establish identity via sequence coverage and assess post-translational modifications (PTMs) at specific amino acid sites. This assay is increasingly implemented for multi-attribute monitoring (MAM) and product release testing, which means that it must be reliable and robust. The data generated from peptide mapping is already relatively complex when one considers only the expected digestion components. It is unnecessarily complicated by missed cleavages (under-digestion), non-specific cleavages (over-digestion), and autolysis peaks (self-digestion of the enzyme) which occur as a function of enzyme:protein ratio, time, temperature, and pH of the sample digestion.

To combat these complexities of digestion, Waters has launched a new in-solution trypsin that addresses these analytical challenges that arise from inefficient digestion. RapiZyme™ Trypsin is a homogeneously methylated recombinant porcine trypsin which is thermally stable and carefully derivatized so as to be extremely resistant to autolysis. This is demonstrated by incubating RapiZyme Trypsin and another industry-leading MS-grade trypsins overnight at elevated temperature (Fig 1).

Here we demonstrate the utility of RapiZyme Trypsin in protein digestion compared to another industry-leading MS grade trypsin in a variety of flexible digestion protocols.

Key Benefits:

- **Autolysis resistance unlocks ability for high enzyme:protein ratio for rapid 30-minute digestion without need for high temperature**
- **Clean chromatographic baselines, decreased need for investigation of unmatched peaks**
- **Demonstrated versatility with a number of unique digestion protocols**

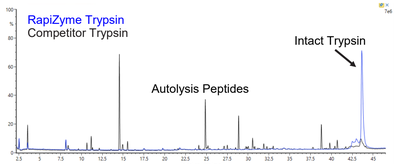
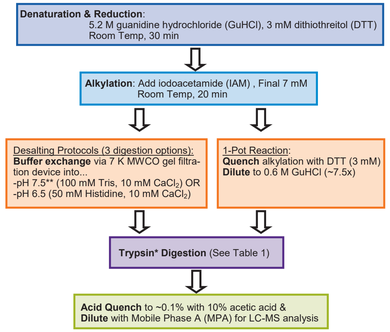


Figure 1. Figure 1. TIC overlay for enzyme blanks for a mock 1:5 enzyme:protein ratio sample with RapiZyme trypsin (blue trace) and another industry-leading competitor trypsin (black trace). These mock samples represent enzyme blanks..

METHODS

mAb Digestion (4 Flexible Protocols)



Condition	Enzyme : Protein Ratio	Temperature	pH	Incubation Time
Accelerated	1:5 (w/w)	37 °C	7.5	30 minutes
Traditional	1:20 (w/w)	37 °C	7.5	1-3 hour
Overnight	1:100 (w/w)	Ambient	6.5	Overnight (15-18hr)
1-Pot (No Desalt)	1:5 (w/w)	37 °C	7.5	2 hours

Table 1. Overview of Digestion Conditions

***RapiZyme Trypsin P/N 186010108**
****Tris $CaCl_2$ Buffer Salts (P/N 186010111)**

LCMS Peptide Mapping Analysis

Peptide mapping experiments were carried out using an ACQUITY™ Premier Peptide CSH™ C18 Column (1.7µm, 2.1 x 100 mm) maintained at 60 ºC for a 50-minute linear gradient of 1-35 %B (80-minute total time). Mobile phase A (MPA) was 0.1% formic acid in water and mobile phase B (MPB) was 0.1% formic acid in acetonitrile. DIA MS detection was performed using Xevo™ G3 QTof MS (MS^E) and BioAccord™ Systems. Data analysis was carried out via UNIFI™ peptide mapping workflow within waters_connect™ Informatics Platform.

Xevo G3 QTOF System

BioAccord System

RESULTS AND DISCUSSION

Each of the investigated protocols (see Table 1) in this study was compared to a “standard protocol” (gray bars) consisting of denatured, reduced, alkylated, and desalted infliximab which was digested with another leading MS-grade trypsin in a 1:20 w/w E:P ratio for 3 hours at 37 ºC. Results for sequence coverage, missed cleavage, trypsin autolysis, and TIC purity are shown below.

For sequence coverage (Fig 2), > 95% is observed in all conditions tested, consistent with the standard protocol. The % TIC response comprised of expected tryptic peptides is representative of the overall purity of the TIC. “Impurities” are missed cleavages, trypsin autolysis species, or other unknown peaks.

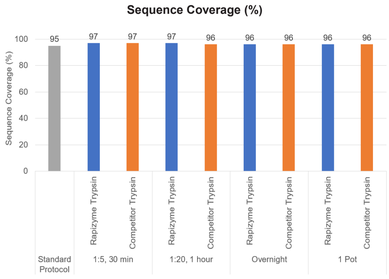


Figure 2. Summary of observed sequence coverage (%) across protocols. A peptide is considered a match if < 10ppm mass accuracy, with at least 2 b/y ions detected. All conditions provide coverage > 95%.

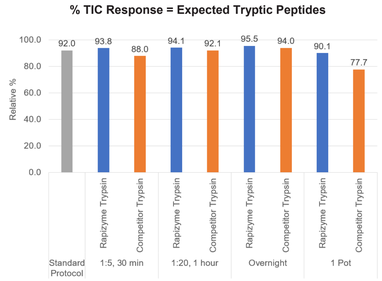


Figure 3. Summary of % TIC area response comprised of expected tryptic infliximab peptides observed in each digest condition.

Regarding missed cleavages (Fig 4), RapiZyme Trypsin contains 2-3% less missed cleavage species in all protocols except the 1-Pot digestion (~7% less), suggesting RapiZyme Trypsin may be more robust in the presence of guanidine hydrochloride at this concentration. As expected, the level of trypsin autolysis species (Fig 5) is higher for the 1:5 E:P ratio digestions for the leading MS-grade trypsin, while the RapiZyme Trypsin samples showed no detectable levels of autolysis species.

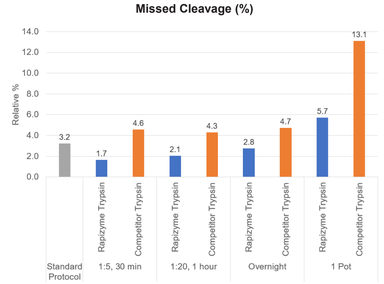


Figure 4. Summary of % missed cleavage across all protocols. RapiZyme Trypsin outperforms the competitor in all conditions.

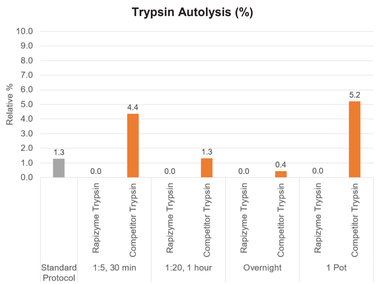


Figure 5. Summary of % trypsin autolysis across all protocols. No autolysis is detected in any of the RapiZyme Trypsin digest conditions.

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Utility of Autolysis Resistant Trypsin in High Enzyme : Protein Ratio Digestion

Since RapiZyme Trypsin is extremely resistant to autolysis, it was tested at a higher E:P ratio of 1:5 with 30-minute incubation time. A significant difference in the quality of resulting data is observed (Figure 2). Significant differences in undesired species are highlighted with red (autolysis) and purple (missed cleavage) arrows. ~94% of the total TIC area in the RapiZyme Trypsin digestion is comprised of expected tryptic cleavage infliximab peptides, while the competitor was only 88% (Fig 6).

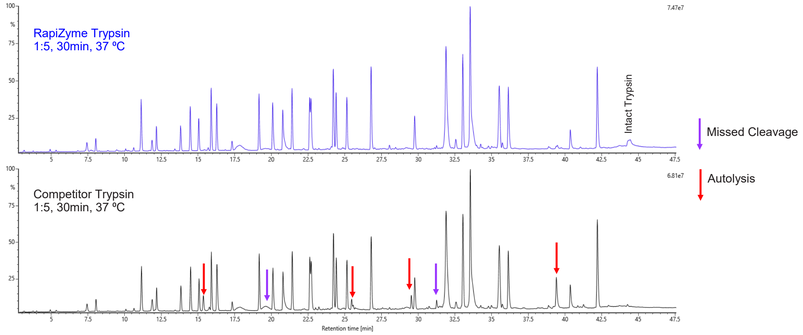

Figure 6. Accelerated Digestion. Comparison of TIC traces for accelerated 30-min digestions with RapiZyme Trypsin and a leading competitor MS-grade trypsin, generated using the Xevo G3 QTof MS. Significant differences in undesired missed cleavage and trypsin autolysis species are highlighted with orange and red arrows, respectively.

Comparable TIC Profile Between Protocols

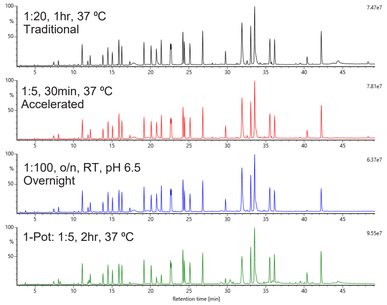

Figure 7. Summary of digestion conditions—Stacked TIC chromatograms showing high level of similarity between all four digestion conditions performed for Remicade using RapiZyme Trypsin.

CONCLUSION

- **Peptide mapping is, and will continue to be, one of the most data-rich assays used for biopharmaceutical characterization, and sample preparation methods are critical in providing clean, clear datasets.**
- **RapiZyme Trypsin, a novel homogenously methylated porcine trypsin, has proven utility to generated reproducible high quality peptide mapping data, due to its high activity and autolysis resistance.**
- **RapiZyme Trypsin can be used flexibly in a variety of protocols, from accelerated digestions, overnight and simple 1 -pot digestion**

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