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Note d'application

Versatile and Rapid Digestion Protocols for Biopharmaceutical Characterization Using RapiZyme™ Trypsin

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Abstract

Complete and clean trypsin digestion is achieved with an enzyme that provides a balance of missed cleavage, non-specific cleavage, and trypsin autolysis. RapiZyme trypsin is a new homogeneously methylated, recombinant porcine trypsin which exhibits excellent thermal stability and improved levels of autolysis resistance. This application note evaluates RapiZyme trypsin in traditional peptide mapping protocols and also explores its applicability in a variety of alternative protocols utilizing various enzyme ratios, pH, incubation time, and temperatures. It was observed that RapiZyme trypsin uniquely enables the use of a high enzyme:protein (E:P) ratio (1:5, w/w) along with a rapid 30-minute digestion. The result of which is a high quality, exemplary peptide map that shows little to no autolysis peak interference despite the use of a high concentration of protease. Additionally, the use of RapiZyme trypsin has made it possible to accelerate traditional E:P ratio digestions (1–3 hours, 1:20 E:P). Furthermore, for those who prefer to digest overnight, RapiZyme trypsin is also demonstrated here to provide a complete, low artifact digestion with conditions based on a 1:100 E:P ratio, pH 6.5 buffer, and an ambient temperature incubation. Finally, this application work expands once more to consider a one-pot reaction condition, wherein RapiZyme trypsin was used in the presence of low amounts of guanidine HCl in a manner not possible with other industry-leading MS-grade trypsin.

Benefits

- Improved levels of autolysis resistance unlocks high enzyme:protein ratios that enable rapid and efficient 30-minute digestion protocols without the need for high temperature incubations
- Affords quick and confident decisions during critical data analysis because of clean baselines that minimize the number of unmatched peaks
- Demonstrated versatility with a number of unique digestion protocols
- Reproducible for long-term method success

Introduction

Peptide mapping is an essential assay for biopharmaceutical characterization and monitoring. By digesting an intact protein or monoclonal antibody (mAb) down to the peptide level, it becomes possible to establish protein identity via sequence coverage and investigate site-specific modifications. Peptide mapping is now being implemented as a multi-attribute monitoring (MAM) approach for quality control and release of biotherapeutics, and therefore must be robust and reliable.[1] Peptide mapping data analysis is quite complex even when thinking only about the expected peptide contents. It is further complicated when the proteolysis enzyme yields missed cleavage (under-digestion), non-specific cleavage (over-digestion), and autolysis peaks (digestion of itself). Additional peaks increase the time spent on data processing and review. Furthermore, peptide mapping is traditionally associated with long sample preparation times. Long digestion times increase sample turnaround time, possibly delaying critical decisions.

To combat these complexities, Waters™ has launched a new in-solution trypsin. 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 and another industry-leading MS-grade trypsin overnight at elevated temperature. As shown in Figure 1, RapiZyme trypsin remains almost entirely intact, while the competitor enzyme exhibits significant autolysis. This application note showcases the use of RapiZyme trypsin first in traditional digestions, then explores its applicability to a variety of other approaches to peptide mapping sample preparation.

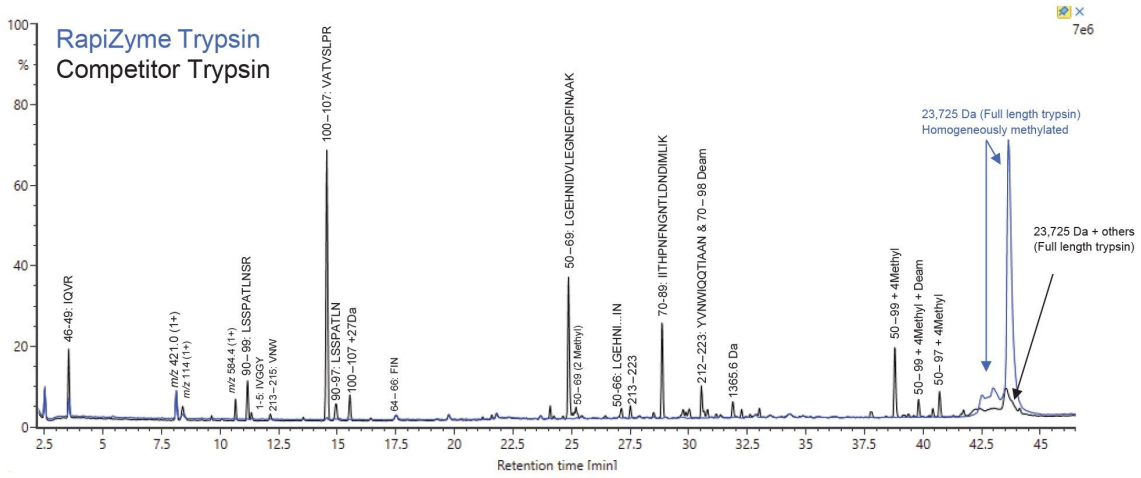

Figure 1. TIC overlay for a mock 1:5 enzyme:protein ratio sample with RapiZyme trypsin (blue trace) and another industry-leading MS-grade trypsin (black trace). These mock samples represent enzyme blanks.

Experimental

Traditional Sample Preparation (Desalt Before Trypsin Digestion)

Remicade™ (infliximab) samples were denatured and reduced in a 5.2 M guanidine hydrochloride solution containing 3 mM dithiothreitol (DTT) for 30-minutes at room temperature. Iodoacetamide (IAM) was then added to a final concentration of 7 mM iodoacetamide (IAM) and incubated for 20-minutes at room temperature. Samples were buffer exchanged via 7K MWCO gel filtration device into pH 7.5 or pH 6.5 digestion buffers. The pH 7.5 buffer was Tris $CaCl_2$ Buffer Salts (p/n: 186010111 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010111-quick-prep-tris-cacl2-buffer-salts-ph-75-4-pk.html>), and the pH 6.5 digestion buffer was 50 mM Histidine, 10 mM $CaCl_2$, prepared in-house. RapiZyme trypsin (p/n: 186010108 < https://www.waters.com/nextgen/global/shop/standards--reagents/186010108-rapizyme-tryspin-ms-grade-4-pk.html>) was added to each sample and incubated as outlined in Table 1. At each timepoint, sample was acidified with 10% acetic acid (final concentration 0.1%) and further diluted with mobile phase A for analysis via LC-MS.

Condition	Enzyme : protein ratio	Temperature	pH	Incubation time	Buffer composition
Accelerated	1:5 (w/w)	37 °C	7.5	30 minutes	100 mM Tris, 10 mM $CaCl_2$ (Waters p/n 186010111)
Traditional	1:20 (w/w)	37 °C	7.5	1–3 hour	100 mM Tris, 10 mM $CaCl_2$ (Waters p/n 186010111)
Overnight	1:100 (w/w)	Ambient	6.5	Overnight (15–18hr)	50 mM Histidine, 10 mM $CaCl_2$
Dilution method (no desalting)	1:5 (w/w)	37 °C	7.5	2 hours	100 mM Tris, 10 mM $CaCl_2$ (Waters p/n 186010111)

Table 1. Flexible trypsin digestion conditions.

One Pot (No Desalt) Samples Preparations

To facilitate a dilution-based one pot digestion protocol, samples were added directly to solid guanidine hydrochloride for a final denaturant concentration of 5 M. They were reduced and alkylated as described above. The samples were diluted to 0.6 M guanidine hydrochloride with the same pH 7.5 digestion buffer (Tris $CaCl_2$ Buffer Salts (p/n: 186010111 <https://www.waters.com/nextgen/global/shop/standards--reagents/186010111-quick-prep-tris-cacl2-buffer-salts-ph-75-4-pk.html>) and trypsin was added at a ratio of 1:5 (w/w) for digestion. After incubation for two hours at 37 °C, samples were acidified with 10% acetic acid (final concentration 0.1%) and further diluted with mobile phase A for analysis via LC-MS.

LC Conditions

LC system:	ACQUITY™ UPLC™ I-Class PLUS
Detection (optical):	ACQUITY TUV
Vials:	QuanRecovery™ Vials with MaxPeak™ HPS (p/n: 186009186)
Column(s):	ACQUITY Premier Peptide CSH™ C_{18}, 130 Å, 1.7 µm, 2.1 x 100 mm (p/n: 186009488)
Column temperature:	60 °C

Sample temperature:	6 °C
Injection volume:	5–10 µL (1–2 µg on column)
Flow rate:	0.2 mL/min
Mobile phase A:	0.1% (v/v) Formic Acid in Water
Mobile phase B:	0.1% (v/v) Formic Acid in Acetonitrile
Gradient:	Initial hold at 1% B for 1 minute, 1–35% B over 50 minutes, 35–85% B over 6 minutes, 85% B for 4 minutes, 85–1% B over 6 minutes, hold at 1% B for 13 minutes

MS Conditions

MS system:	ACQUITY RDa™
Ionization mode:	ESI+, Full Scan MS with Fragmentation
Acquisition range:	*m/z* 50–2000
Capillary voltage:	1.2 kV
Collision energy:	60–120 V (low/high energy ramping)
Cone voltage:	30 V
Desolvation temperature:	350 °C

Intelligent data capture:	On

Data Management

LC-MS caquisition & processing:	UNIFI™ v 3.0.0.6 operated under waters_connect™ v 2.1.0

Results and Discussion

The purpose of this study was to evaluate the performance of RapiZyme trypsin and its use in various types of protocols. It was also of interest to compare its performance to that of another industry leading MS-grade trypsin which boasts of autolysis resistance. Key parameters constituting a successful digestion include low levels of each of the following components: 1) missed and non-specific cleavages, 2) trypsin enzyme autolysis species, 3) unmatched/unknown peaks, and 4) artificial post-translational modifications (PTMs), such as deamidation or oxidation that can result from nonoptimal digestion conditions.[2–3] All of these create added complexity in the data, which puts an extra burden on the analyst. If unmatched peaks are present after standard peptide searching against an *in-silico* digest, the user would need to widen the search to include parameters such as semi-tryptic digestion (to assign non-specific cleavage), an increased number of missed cleavages, and the trypsin sequence itself (along with its derivatization) to begin matching autolysis peaks. Each of these additional parameters lengthens the time it takes the software to process the data, and the amount of time a user must spend on the review.

Accelerating Traditional Trypsin Digestion

Many reference papers outlining the tryptic digestion of mAbs have called for the use of a denatured, reduced, and alkylated protein sample that gets subjected to a desalting step prior to digestion.[1–7] In these procedures, trypsin has thereafter been applied at an enzyme:protein ratio of 1:20–1:25 (w/w) and incubations have been performed for up to four hours at an elevated temperature. Such trypsin digestion protocols have been adopted by many biopharmaceutical industry labs as part of the peptide mapping analytical workflow. This is where we began the evaluation of RapiZyme trypsin. Using Remicade (an IgG1 kappa mAb) as a case study, we compared

the performance of RapiZyme trypsin to another leading competitor trypsin, at an E:P ratio of 1:20, incubated for three hours at 37 °C. Detailed analyses were then performed by LC-MS. Comparable performance was observed (Figure 2, TIC traces shown) for RapiZyme trypsin and the competitor trypsin when three hours of incubation were applied. Each give >93% sequence coverage, and more than 95% of the integrated TIC area is comprised of Remicade peptides with expected tryptic cleavages. No significant difference in deamidation or oxidation levels was observed upon comparing the digestions.

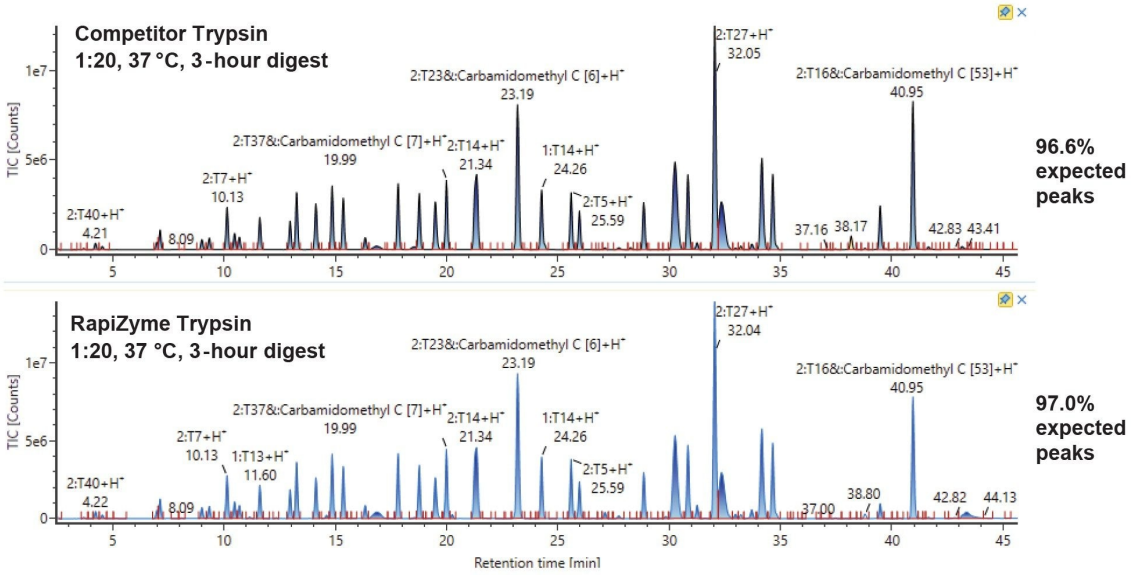

Figure 2. TIC chromatograms for 3-hour 1:20 enzyme:protein ratio digestions of Remicade with another industry-leading trypsin (black trace) and RapiZyme trypsin (blue trace), showing equivalent results.

Since the three hour digestion with a 1:20 E:P ratio gave near complete digestion of the mAb, we decided to test a shorter incubation time with the same conditions. The overlay in Figure 3 demonstrates excellent comparability for the one hour (blue trace) and three hour (black trace) digestions of Remicade, indicating that it is possible to achieve equivalent results in only one hour when RapiZyme trypsin is applied.

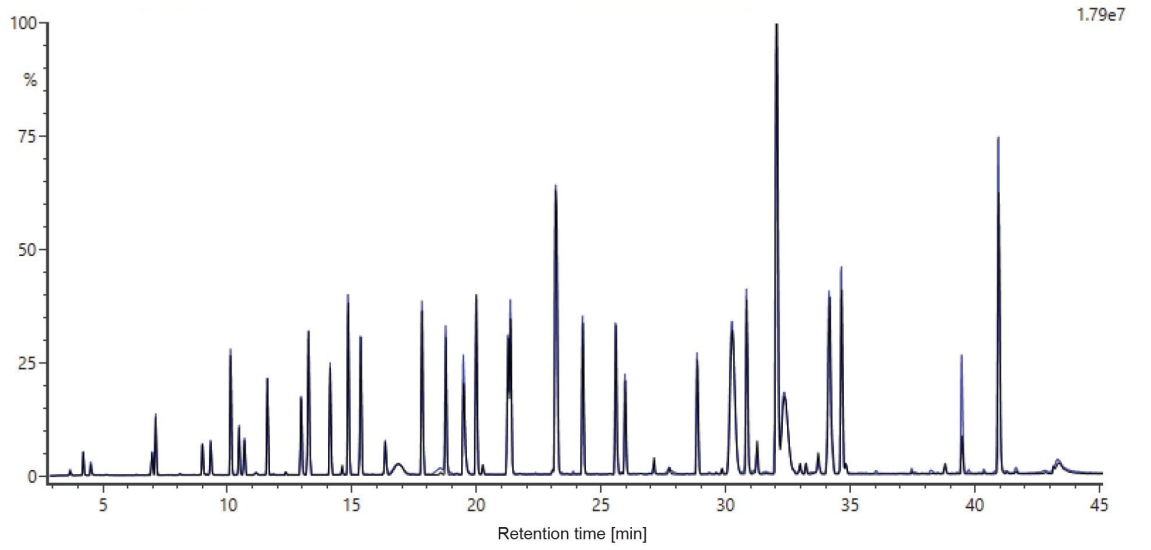

Figure 3. TIC chromatograms for a 3-hour (black trace) 1:20 ratio digestion of Remicade with RapiZyme trypsin, achieving the same level of digestion in only 1 hour (blue trace) with same conditions.

In peptide mapping sample preparation, reproducibility is of utmost importance. The repeatability and batch to batch reproducibility of the one hour RapiZyme trypsin digest of Remicade was further evaluated using two different batches of RapiZyme trypsin, each represented with three digestion replicates for each batch. Figure 4 displays the TIC overlay for these six injections, and it can be seen that there is excellent reproducibility, similar levels of digestion completeness and TIC patterns for the expected peptides. The total amount of missed cleavage for the study was 0.55% ± 0.3%. Overall, 97.5% ± 0.3% of the TIC area is made up of fully tryptic peptides derived from the Remicade light and heavy chain sequences.

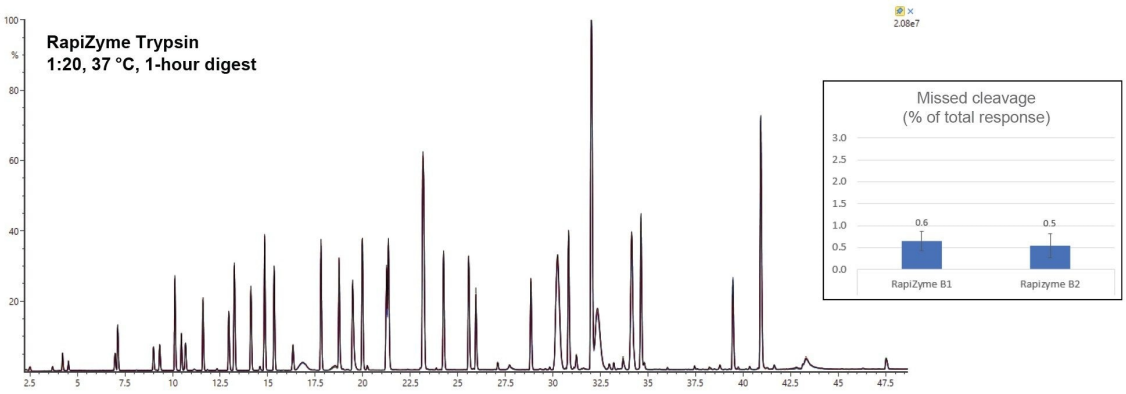

Figure 4. TIC overlay for 3 digestion replicates each for two different batches of RapiZyme trypsin as obtained with 1:20 enzyme:protein digestions. Inset shows a bar graph comparison of % missed cleavage for each batch of RapiZyme & trypsin (replicates n=3).

Can We Go Even Faster?

In biopharmaceutical development, there is an ongoing search for ways to improve both workflow efficiency and sample turnaround time. At the same time, there are efforts made to minimize artifactual modifications that arise from longer digestion times.[3–4] The novelty of RapiZyme trypsin lies specifically in its stability, autolysis resistance, and high activity. This allows more flexibility to use higher enzyme-to-protein ratios and the chance to explore shorter digestion times along with the use of a wider range of temperatures. For Remicade, we opted to test a 1:5 ratio for 30-minutes at a standard incubation temperature of 37 °C. The resulting labeled TIC chromatograms for RapiZyme trypsin vs another industry-leading trypsin are shown in Figure 5. There is a substantial difference in the quality of the digestions, mostly due to peptides generated from trypsin autolysis during the digestion when using the competitor enzyme at a higher E:P ratio. The zoomed section in Figure 5 highlights a section with a significant amount of autolysis species (indicated with red arrows). RapiZyme trypsin did not show any appreciable signal for interference peaks in this retention window, nor in any others where Remicade peptides elute. RapiZyme trypsin provided a fast, clean digestion with >93% sequence coverage, <1% missed cleavage, and <0.1% trypsin autolysis. (Relative abundance (%) of missed and non-specific cleavage are calculated via MS response of these species compared to the total MS response for all Remicade-derived peptides, in a manner consistent with previously published work.)[2]

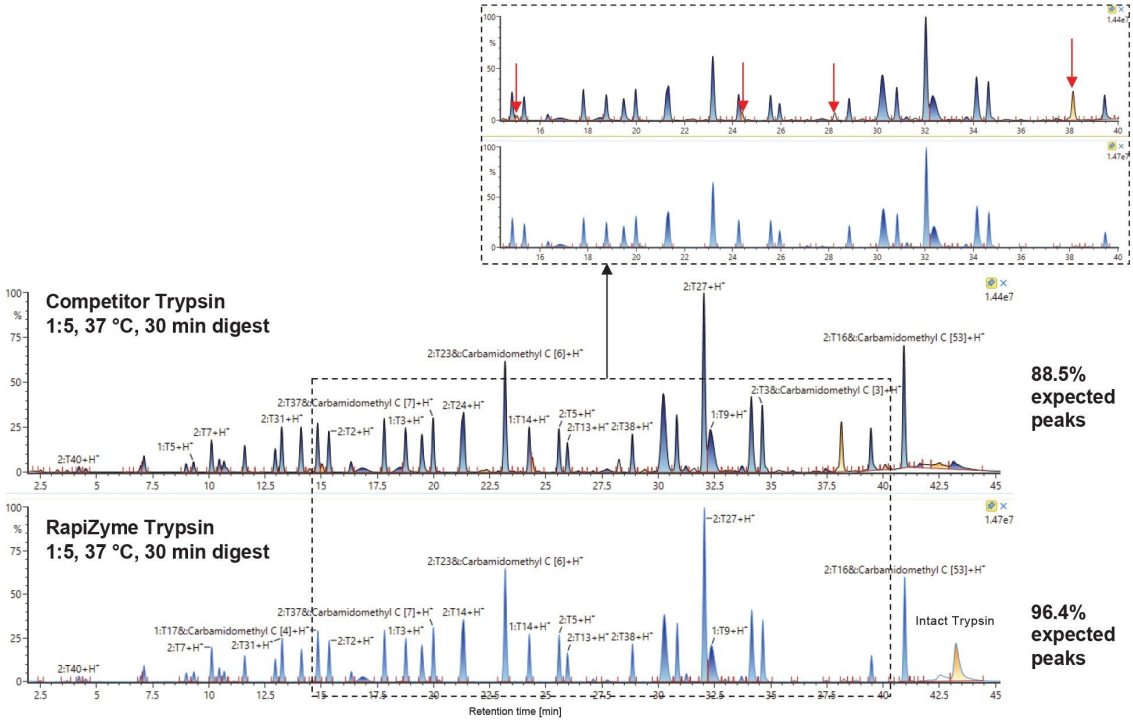

Figure 5. Comparison of 1:5 digests, another industry-leading competitor (top panel) vs RapiZyme trypsin (bottom panel), with zoomed section of retention time window 14 to 40 min. Red arrows highlight trypsin autolysis and unknown peaks.

The same repeatability and batch-to-batch reproducibility exercise from above was applied to this accelerated digestion condition. Figure 6 shows the TIC overlay of six samples corresponding to two batches of RapiZyme trypsin, each represented with three digestion replicates. Again, excellent digestion reproducibility and overall completeness of digestion were observed. Overall, 96.3% ± 0.6% of the TIC area is made up of fully tryptic peptides derived from Remicade.

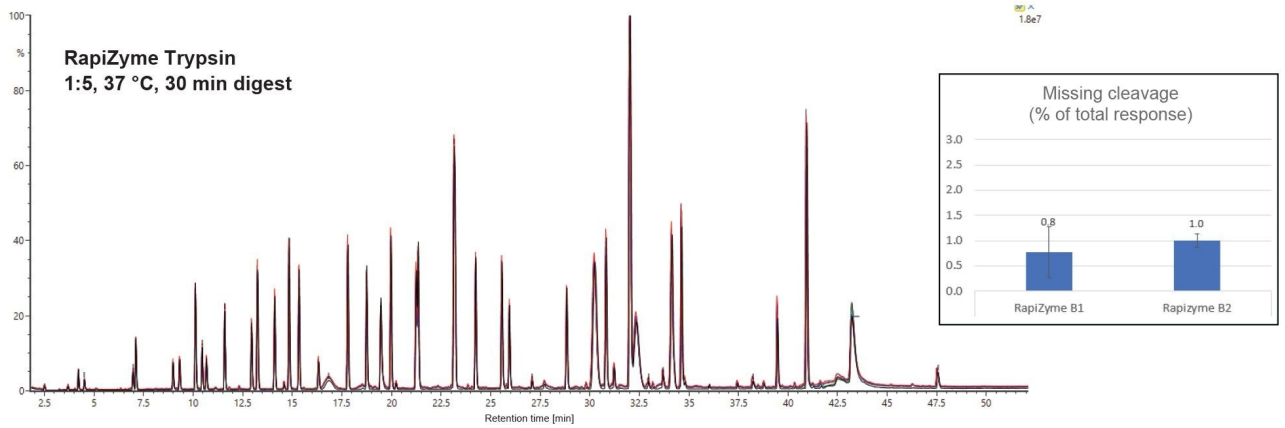

Figure 6. TIC overlay for 3 digestion replicates each for two batches of RapiZyme trypsin as obtained with 1:5 enzyme:protein digestions. Inset shows a bar graph comparison of % missed cleavage for each batch of RapiZyme trypsin (replicates n=3).

What About Improving Overnight Digestion?

While many scientists desire faster digestions workflows, there are others who prefer the flexibility of setting up and returning to an overnight digestion. There are a few considerations to take into account for overnight digestions. First, RapiZyme trypsin is quite active, so the E:P ratio and temperature should be optimized to control any non-specific cleavage (over-digestion) that may arise. The other consideration is PTMs, such as deamidation, that occur due to digestion conditions, namely neutral-to-high pH and elevated temperatures.[3] Unfortunately, this is where most trypsin enzymes retain their highest activity. In order to mitigate these concerns in our overnight digestion evaluation with RapiZyme trypsin, we chose to use a low 1:100 enzyme ratio, ambient temperature conditions (20–25 °C), and a pH of 6.5 buffer. These parameters provide a clean complete overnight digestion, as shown in the TIC in Figure 7A. We observe >90% sequence coverage with a < 1% level for missed cleavages, non-specific cleavages, and unknown peaks combined. Very low levels of overall deamidation and oxidation are observed. To demonstrate the effects of temperature and pH with overnight incubation, an additional digestion of Remicade was performed with the 1:100 E:P ratio, overnight time interval but with a pH 7.5 buffer and 37 °C incubation. Select deamidation-prone peptides were compared between 1) the accelerated 30-minute digestion described previously, 2) the overnight pH 6.5 ambient temperature digestion, 3) and the overnight pH 7.5, 37 °C digestion. The extracted ion chromatograms (Figure 7B) for heavy chain peptide T27 (VVSVLTVLHQDWLNGK) show equivalently low levels of deamidation for the 30-minute and pH 6.5 optimized

overnight digestions. Meanwhile, > 20% deamidation was observed for this peptide with the pH 7.5, overnight 37 °C condition. This verifies that the optimized overnight protocol does not introduce deamidation artifacts. Overall, the 1:100 enzyme ratio pairs with a pH 6.5 buffer and room temperature overnight incubation to provide a third alternative for generating clean and complete digestion with RapiZyme trypsin.

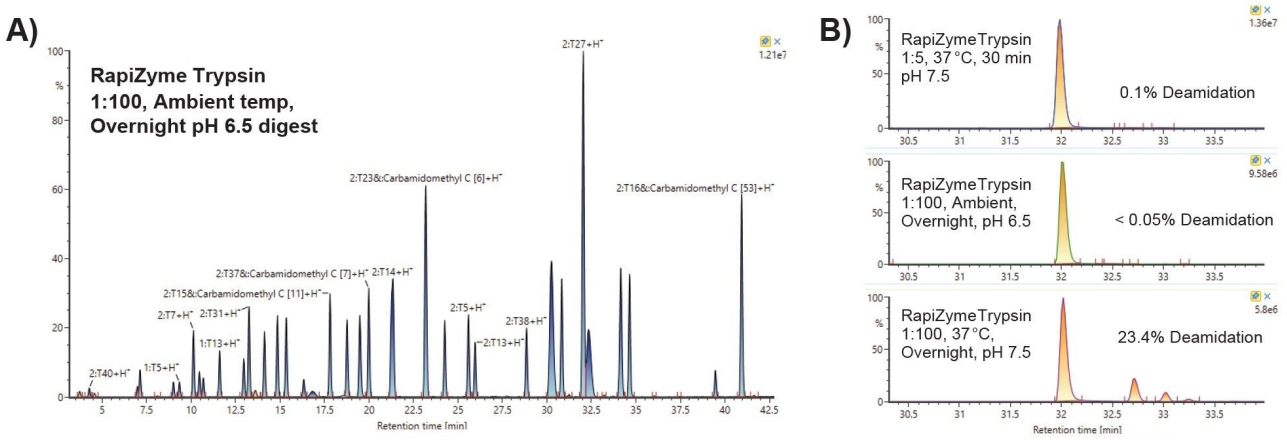

Figure 7. A) Labeled TIC for Remicade 1:100 Overnight pH 6.5 digestion. B) Extracted ion chromatograms for deamidation-prone peptide HC T27 (VVSVLTVLHQDWLNGK), m/z 603.6 (3+) and 904.4 (2+), for 30 min and overnight incubations with a pH 6.5 versus 7.5 buffer.

Can We Skip The Desalting Step?

All of the protocols discussed up to this point used samples in which a desalting cartridge is employed to remove guanidine hydrochloride denaturant and reduction/alkylation reagents prior to trypsin digestion. Most trypsins are greatly hindered by common protein denaturants like guanidine hydrochloride and urea.[2] However, many analysts would desire a protein digestion protocol that does not include the desalting step, for a variety of reasons. An inherent risk in using desalting cartridges is the variability in protein recovery after buffer exchange. Most commercially available devices report an average recovery of 70–90%, and it can be highly sample dependent.[8] Eliminating the desalting step promotes more consistency among sample concentrations and digestion conditions. It is also an extra step that adds more time and effort to the sample preparation workflow. Given that RapiZyme trypsin has proven to be reproducible, autolysis resistant, and efficient when applied to the desalted samples, it was worth exploring its possible application to a one pot digestion protocol that skips the traditional desalting step. To facilitate the digestion, a dilution step was incorporated to bring the final guanidine

hydrochloride concentration to 0.6 M after the Remicade sample was reduced and alkylated. RapiZyme trypsin was then added to the sample to give a 1:5 enzyme ratio and the digestion was incubated for 2 hours at 37 °C. The results from this work, as compared to another industry-leading competitor trypsin, are presented in Figure 8. Overall TIC area for the RapiZyme sample is 96% comprised of full tryptic Remicade peptides, whereas the competitor was only 82% (Figure 8A). This disparity stems primarily from differences in missed cleavages and trypsin autolysis species (Figure 8B & 8C, respectively). The competitor trypsin contained ~10% of the total TIC peak area as missed cleavage, and ~6% as autolysis peaks. This indicates that RapiZyme trypsin can provide a near complete digestion even in the presence of significant concentrations of guanidine hydrochloride. This may offer a viable alternative for those who desire to avoid desalting.

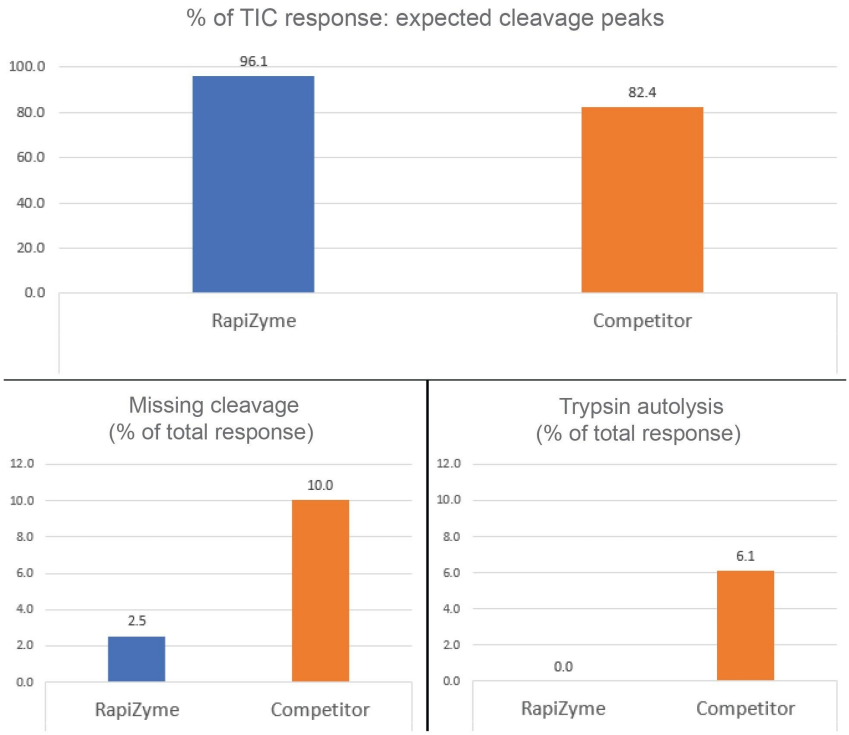

Figure 8. One-Pot (non-desalted) diluted protocol results. Panel A) shows a comparison for % TIC response that is made up of expected cleavage peaks in the 1 Pot digestions of Remicade with RapiZyme trypsin and another industry-leading competitor trypsin. "Impurities" include missed and non-specific cleavages, trypsin autolysis, and unknown peaks. The most significant differences stem from missed cleavage (Panel B) and trypsin autolysis (Panel C).

Conclusion

Peptide mapping is, and will continue to be, one of the most important data-rich assays that can be applied for biopharmaceutical characterization and monitoring. As such, the sample preparation and methods for this assay must be robust and reliable. That said, many labs have a variety of requirements and standard protocols they wish to follow. The method development exercise outlined in this application note shows that RapiZyme trypsin,

with its unique autolysis resistance and digestion efficiency, can be a means to more quickly and more creatively prepare high quality peptide mapping samples. RapiZyme trypsin has proven to be useful in traditional digestions, in rapid 30-minute and flexible overnight digestions, and even in simple one-pot protocols. RapiZyme trypsin provided peptide maps with high levels of similarity and data quality for Remicade peptide mapping with each of the newly envisioned protocols (Figure 9).

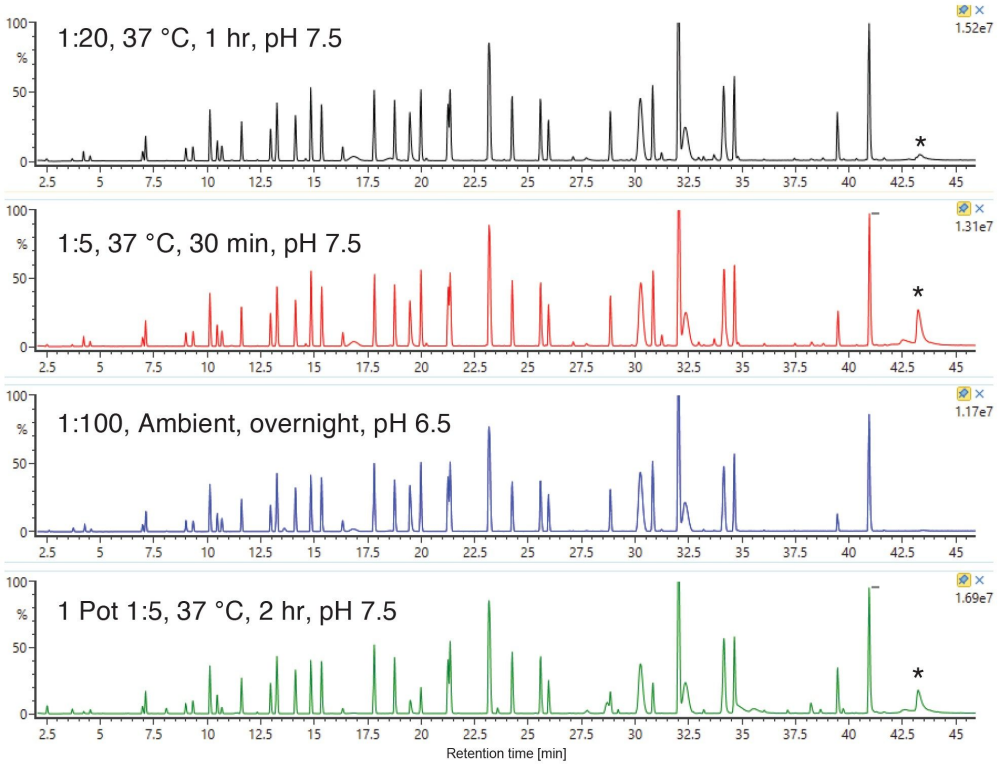

Figure 9. Summary of digestion conditions—Stacked TIC chromatograms showing high level of similarity between all four digestion conditions performed for Remicade (indicates intact trypsin peak).*

References

1. Ranbaduge R, Yu YQ. A Streamlined Compliant Ready Workflow for Peptide-Based Multi-Attribute Method

(MAM). Waters Application Note, 720007094. 2020 Dec.

2. Mouchahoir T, Schiel JE. Development of an LC-MS/MS peptide mapping protocol for the NIST mAb. *Anal and Bioanal Chem*. 2018; 410: 2111–2126.

3. Ren D, Pipes GD, Liu D, Shih L, Nichols A, Treuheit MJ, Brems DN, Bondarenko PV. An Improved Trypsin Digestion Method Minimizes Digestion-Induced Modifications on Proteins. *Anal Biochem*. 2009; 392: 12–21.

4. Millian-Martin S, Jakes C, Carillo S, Buchanan T, Guender M, Kristensen DB, Sloth TM, Oregaard M, Cook K, Bones J. Inter-laboratory Study of an Optimized Peptide Mapping Workflow Using Automated Trypsin Digestion for Monitoring Monoclonal Antibody Product Quality Attributes. *Anal and Bioanal Chem*. 2020; 412: 6833–6848.

5. Evans AR, Hebert AS, Mulholland J, Lewis MJ, Hu P. ID-MAM: A Validated Identity and Multi-Attribute Monitoring Method for Commercial Release and Stability Testing of a Bispecific Antibody. *Anal Chem*. 2021; 93 (26): 9166–9173.

6. Song YE, Dubois H, Hoffman M, Eri SD, Fromentin Y, Wiesner J, Pfenninger A, Clavier S, Pieper A, Duhau L, Roth U. Automated Mass Spectrometry Multi-Attribute Method Analyses for Process Development and Characterization of mAbs. *J Chrom B.* 2021; 1166: 122540.

7. Hao z, Moore B, Ren C, Sadek M, Macchi F, Yang L, Harris J, Yee L, Liu E, Tran V, Ninonuevo M, Chen Y, Yu C. Multi-Attribute Method Performance Profile for Quality Control of Monoclonal Antibody Therapeutics. *J Pharm Biomed Analysis*. 2021; 205: 114330.

8. Instruction Manual for PD-10 Desalting Columns. Cytiva #52130800 BD. Accessed via www.cytivalifesciences.com on 12 Dec 2022.

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