Rapid Peptide Mapping via Automated Integration of On-line Digestion, Separation and Mass Spectrometry for the Analysis of Therapeutic Proteins

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Overview

Purpose: An automated system was developed that integrated rapid trypsin digestion without pretreatment, on-line desalting and high resolution LC/MS/MS.

Methods: A rapid sample preparation and separation system equipped trypsin column was used for fast trypsin digestion of reduced as well as native proteins. This sample preparation instrument was coupled to a hybrid quadrupole-Orbitrap mass spectrometer for peptide mapping experiments and the resulting data sets were analyzed.

Results: For the reduced and alkylated antibody sample, a 100% sequence coverage of the light chain and 91% sequence coverage of the heavy chain were observed. For the untreated/native antibody sample, 90% sequence coverage of the light chain and 73% sequence coverage of the heavy chain were observed. Furthermore, a digest of native serum albumin resulted in 51% sequence coverage. The low coverage percentage value associated with the untreated/native protein analytes can be attributed to disulfide bonded peptides that were likely recovered from the analytical system but not recognized by the proteomics software.

Introduction

Biological systems are extraordinarily dynamic. As such, it is often the case that the time associated with protein sample preparation and analysis delays the detection of malformations until it is too late to take action. Furthermore, a lack of hands-free automation puts an enormous strain on analysts to replicate results across multiple labs. Presented here, is the automated integration of rapid sample preparation, separation and MS used for peptide mapping of a reductively alkylated monoclonal antibody, untreated (native) antibody and untreated human serum albumin (1, 2).

Methods

Samples

To reduce the intact mAb, the sample was incubated for 1 hour at 60 C in 6 M guanidine HCl containing 25 mM DTT for complete reduction. Following reduction the samples was reacted with 100 mM iodoacetic acid for 1 hour at room temperature. Native samples were simply diluted to their final concentration in tris-buffered saline pH 7.4 prior to injection. Peptide mapping of all samples was performed using a modified Dionex ultimate 3000 RSLC nano system system equipped with a Perfinity No Reduction or Alkylation (NORA) Trypsin Column, Digest Buffers from Perfinity, a C18 trap column (Halo, 0.3 X 20 mm) and C18 analytical column (Halo 0.3 X 100 mm).

Online Trypsin Digestion

Untreated antibody solutions were directly injected into the system. Digest efficiency was monitored at various times and temperatures. Simultaneous denaturation and enzymatic digestion were performed at 70 C. Reductively alkylated samples were processed at 50 C. 2 minute digestion times were utilized for all samples.

Chromatography

A Dionex Ultimate 3000 RSLC nano system equipped with a microflow flow selector pumping at 20-50 uL/min was used for the desalting and reversed phase operations. Following digestion, the trapping column was brought in-line with the analytical column by valve switching. Both columns were desalted for 3 column volumes using initial gradient conditions. For peptide mapping experiments, peptides were eluted with a 50 min gradient at a flow rate of 20 µL/min. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). LC gradient was 0-50%B in 50 min.

Figure 1 shows the schematics of the LC configuration used for automated peptide mapping. All transfer tubing was 75 µm Thermo Scientific™ Dionex™ nanoViper™ fingertight fitting system. All valves and columns were operated under isothermal condition in a column oven. An isocratic pumping system was utilized for all sample preparation steps while binary pumps were used for the reversed phase gradient. This configuration enables the digestion of a sample while another sample is separated by reversed phase. By parallel processing in this way a sample can be run every 7 minutes.

Mass Spectrometry

Peptides eluted from analytical column were analyzed using a data-dependent top 10 experiment on the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer (Figure 2). Resolution was 70K for the full MS, 17.5K for HCD MS/MS with a dynamic exclusion of 30 seconds. Detailed instrument parameters are listed in Table 1.

Thermo Scientific™ Proteome Discoverer™ Software revision 1.3 was used to search the protein database with the MASCOT™ search engine for all database searches. The disulfide linked peptides were identified using StavroX (1). Raw files generated by the Q Exactive hybrid quadrupole-Orbitrap mass spectrometer were searched directly using a 10 ppm precursor mass tolerance and a 20 amu fragment mass tolerance.

FIGURE 1. Schematics of the LC configuration for automated peptide mapping.

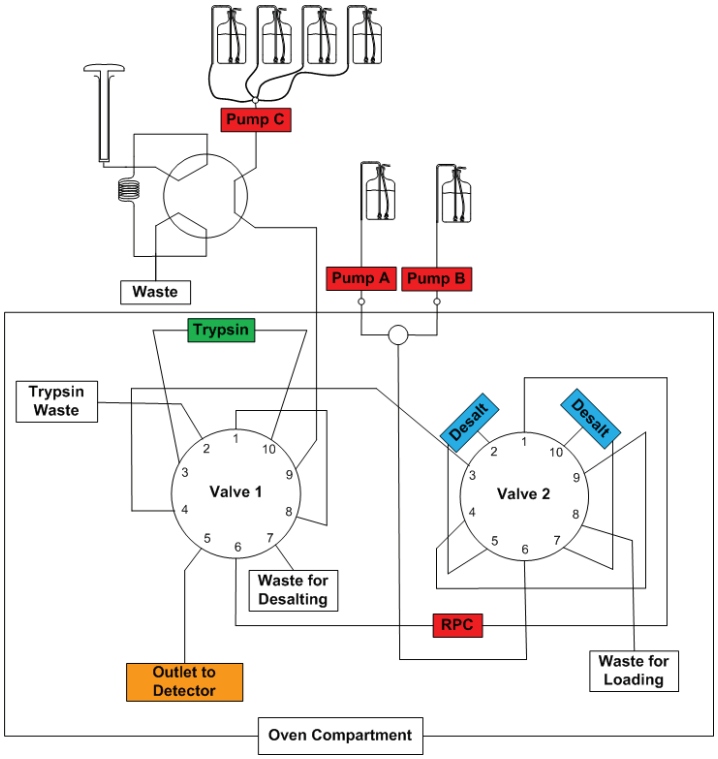


Figure 2. Schematic of the Q Exactive hybrid quadrupole-Orbitrap mass spectrometer.

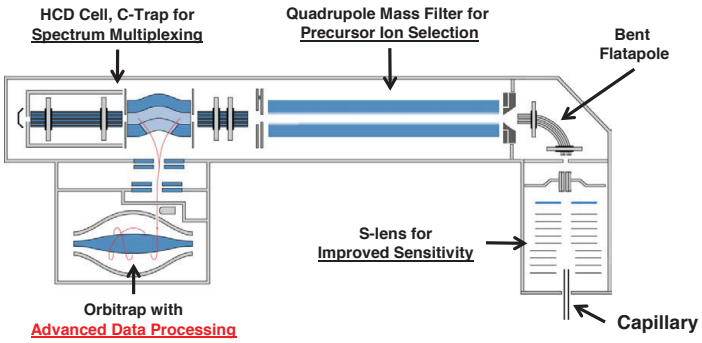


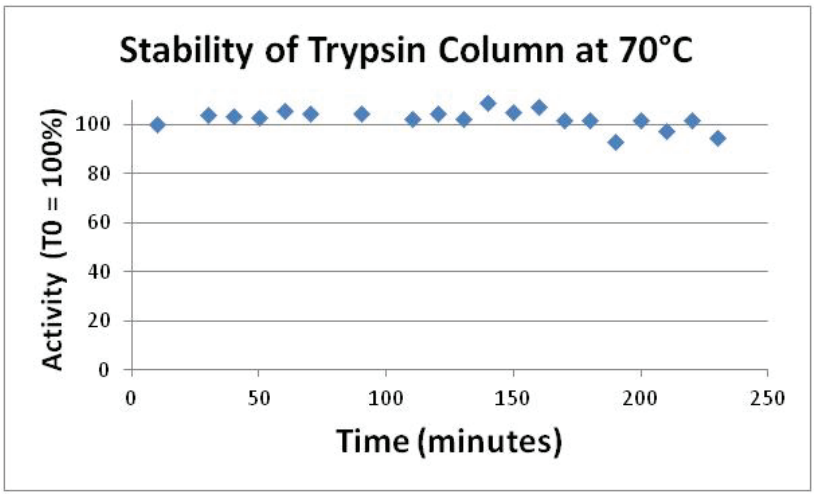
Table 1. The Q Exactive hybrid quadrupole-Orbitrap mass spectrometer instrument method parameters

Parameters	Settings
Full MS scan range	400-2000
MS/MS fixed first mass	100
AGC	1e6, full MS 5e4, MS/MS
Max injection time (ms)	2, full MS 60, MS/MS
Isolation width (m/z)	2.0
NCE	27
Under fill ratio	1%
Dynamic Exclusion	30 sec

Results

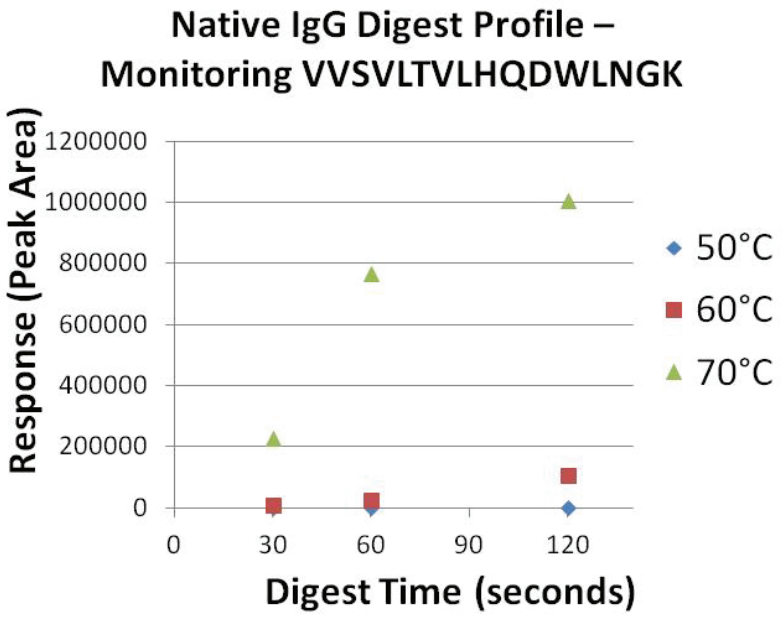
Historically, Kumakura et. Al (2), showed that in the best-case scenario trypsin that was simply immobilized exhibited a 60% reduction of the activity 30 minutes at 70 C. Shown in Figure 3, the proprietary stabilization process of the enzymes three dimensional structure enables prolonged operation under these harsh conditions.

FIGURE 3. Data suggests that a trypsin column was developed capable of operation over prolonged periods at elevated temperatures.



As shown in Figure 4, when the digestions of native antibody samples were performed at various times and temperatures, a dramatic increase in efficiency was observed at 70 C. These results suggest that under these conditions the samples were simultaneously denatured and enzymatically digested. This observation validates results obtained by Vermeer et. al. that showed that the denaturation of antibody variable regions occurs at 60 C and constant region at 70 C (3).

FIGURE 4: Native mAb digested at various times and temperatures.

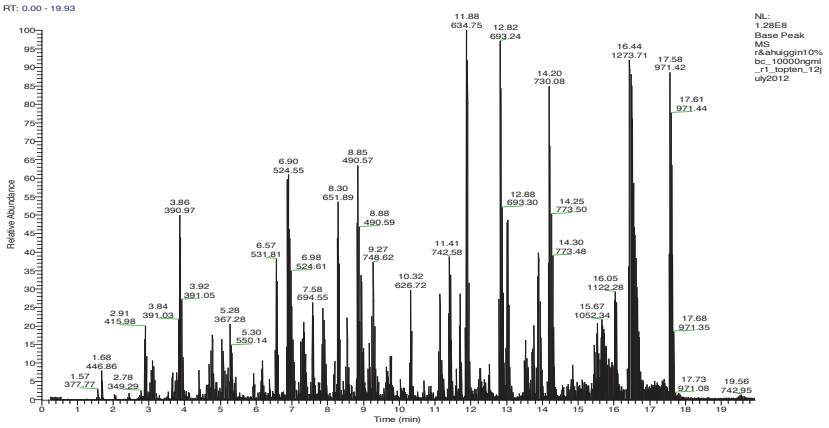


As shown in Table 2, digestion of reduced and alkylated mAb samples yielded sequences coverage exceeding 90%. Even for the samples of digested without pretreatment sequence coverage was 79% for heavy chain and 97% for light chain. The base peak chromatograms obtained for native antibody digestion is shown in Figure 5.

Table 2. Coverage determinations of mAb digests.

Sample	Light Chain	Heavy chain
Reduced and Alkylated	100%	91%
Native	97%	79%

FIGURE 5: Base peak chromatogram of native mAb digestion without reduction and alkylation.



This online digestion experiment was also applied to native human serum albumin. The extensive disulfide linkages in this molecule makes it an especially challenging case for native digestion. Figure 6 shows the coverage determinations of native HSA digests. Figure 7 shows the base peak chromatogram of native HSA digestion without reduction and alkylation. The identification of a large number of canonical peptides suggests native human serum albumin (HSA) was effectively digested. Given the highly bridged nature of HSA this data suggests that many different types of less stable proteins and antibodies can effectively be digested under these conditions.

Since the total digest time for this work was <5 minutes it is possible that this workflow would be extremely useful in situations where close to real time monitoring would be advantageous such as process monitoring. Because reduction and alkylation are not necessary, it is possible that this technology could also be successfully applied to disulfide bond mapping.

TABLE 6. Coverage determinations of native HSA digests.

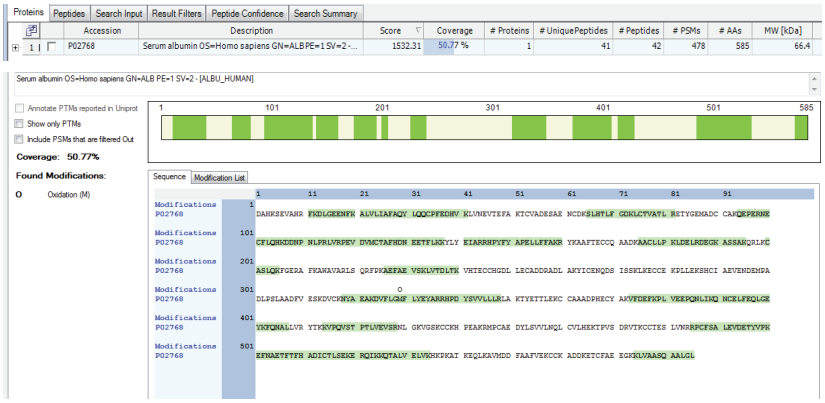
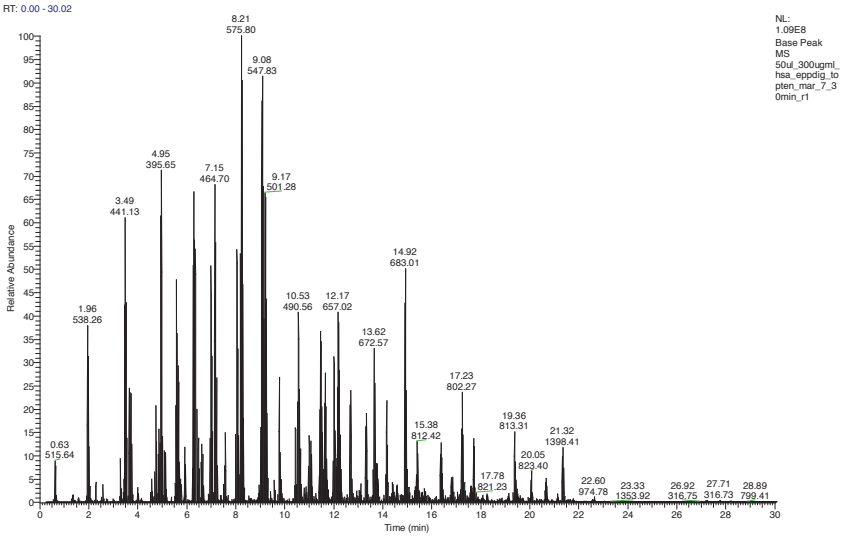


FIGURE 7: Base peak chromatogram of native HSA digestion without reduction and alkylation.



Conclusion

- An automated workflow was developed for protein therapeutics peptide mapping which combined online trypsin digestion and high resolution accurate mass MS.
- A trypsin column was developed capable of operation over prolonged periods at elevated temperatures.
- Operation under denaturing conditions enabled rapid digestion without pretreatment.
- Direct coupling of this system to the Q Exactive Orbitrap hybrid quadrupole-Orbitrap mass spectrometer provided confident amino acid sequence information for the monoclonal antibody.
- A confident peptide mapping experiment including online trypsin digestion and Orbitrap LCMS/MS analysis was achieved within a hour.
- Potential future applications include use of this set-up in fast diagnostics, process monitoring and disulfide bond mapping.

References

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