Comprehensive Sequence and Post-translational Modifications Analysis of Monoclonal Antibody by Flash Digest and LC-High Resolution MS

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Overview

Purpose: To develop a workflow for fast and comprehensive characterization of peptide sequence, identification and relative quantitation of post-translational modifications (PTMs) of monoclonal antibody (mAb).

Methods: Native and oxidatively stressed IgG mAb were enzymatically digested by Flash Digest™ kit with trypsin. Peptide samples were analyzed by online LC-MS on a Thermo Scientific™ Q Exactive™ mass spectrometer. Peptide sequence mapping, identification and quantification of PTMs were performed by Thermo Scientific™ PepFinder™ software (version 1.0).

Results: Comprehensive and simultaneous sequence and PTM analysis with a particular focus on oxidation of IgG mAb was realized by combining rapid digestion, high resolution, accurate mass (HRAM) data and PepFinder software. This workflow greatly shortens the sample preparation and data analysis time while providing great sensitivity to detect low level PTMs.

Introduction

As well established and fast growing biotherapeutics, mAbs have been approved for the treatment of diseases such as cancer, inflammatory, infectious and autoimmune diseases etc.[1,2] To ensure product efficacy and safety, the quality of biotherapeutics needs to be closely monitored. Various analytical methods have been used to study quality attributes such as structural integrity, aggregation, glycosylation pattern or amino acid degradation. Here, we report a fast and sensitive approach by combing fast enzymatic digestion, high resolution mass spectrometry and user friendly new data processing software for sequence and post translational modifications analysis. This approach provides an effective way to characterize protein therapeutics in bioprocess development.

Methods

Sample Preparation

Differential oxidative stress was induced by 5 mM hydrogen peroxide and quenched by the addition of 1mM sodium thiosulfate at various time points. The native and oxidatively stressed IgG samples were trypsin digested using a Flash Digest kit (Perfinity Biosciences Inc). Digestion time was optimized by incubating native, non-reduced IgG mAb at 70 °C for various durations from 15 to 120 minutes. One portion of the digest was analyzed by online UHPLC-ESI-MS/MS. The other portion was reduced and alkylated before similar analysis. All chemicals were purchased from Sigma Aldrich unless it is specified.

Liquid Chromatography

Native and Oxidatively stressed tryptic peptide samples were analyzed on Thermo Scientific™ Dionex™ UltiMate™ 3000 XRS system and OAS autosampler coupled to the Thermo Scientific™ Q Exactive™ MS. Peptides were separated on an ACQUITY® BEH 130 C18 column (2.1x100mm, 1.7μm, Waters) with column temperature set as 40 °C at a flow rate of 300 μL/min with solvent A (0.05% trifluoroacetic acid in H_2O) and solvent B (0.045% trifluoroacetic acid in acetonitrile).

Injection amount: 8.0 μg digested protein on column

Time [min]	Flow [μL/min]	Mixture [%B]
0	300	0.1
5	300	0.1
94	300	35
94.5	300	95
99.5	300	95
100	300	0.1
110	300	0.1

Mass Spectrometry

The Q Exactive MS interfaced with H-ESI II ion source was employed for MS analysis. Acquisition method was set with full scan (resolution 70,000 at FWHM *m/z* 200) and top 5 data dependent MS/MS (17,500 resolution) in positive mode.

=== HESI Source: ===	
Spray Voltage (+)	3800V
Capillary Temperature (+)	320°C
Sheath Gas (+)	40
Aux Gas(+)	10
Sweep Gas(+)	0
Heater Temperature (+)	300°C
S-lens	50

Full MS Scan in positive mode: Resolution=70,000; AGC=3e6; IT=100ms; Scan range=*m/z* 300-1800; Lock mass=off; Microscans=1

Top 5 data dependent MS/MS: Resolution=17,500; AGC=1e5; IT=250ms; NCE=27; Isolation window=*m/z* 2; Fixed first mass=*m/z* 130

Data Analysis

The mapping of mAb sequence, disulfide linkages and identification of PTMs are performed in PepFinder software. PepFinder software is designed for in-depth characterization of biotherapeutic proteins. It offers automatic workflow for identification of disulfide bonds, glycopeptides and other PTMs, i.e. oxidation, deamidation etc by mono-isotopic mass at MS level and confirmation by MS/MS fragments indicated with a confidence score. The peptide sequence coverage map with color code for signal intensity of each characterized peptide and modification summary report with relative quantitation percentage are generated on the user friendly interface. For unknown /untargeted modifications, the amino acid sites are indicated with accurate mass of the modification for further interpretation.

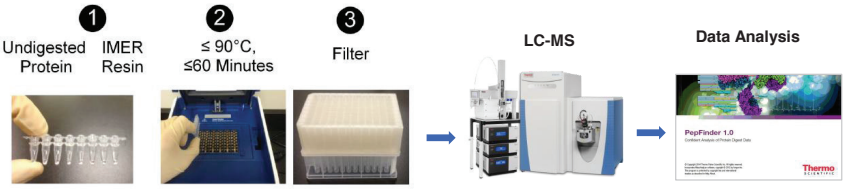
Results

Tryptic Digestion Time Optimization of mAb by Flash Digest Kit

Flash Digest is a very active, highly stable immobilized trypsin reactor that is combined with heating technology for fast reproducible digestions. The trypsin column makes use of a high concentration of trypsin while simultaneously eliminating autolysis in order to push the non-complete digestion due to the decrease of substrate concentration near the completion of digestion reaction.

Using the Flash Digest kit (below workflow 1-3), digestion time was optimized by incubating native, non-reduced IgG mAb at 70 °C at 15, 30, 45, 60, 75, 90, 105 and 120 min. The filtered samples were directly subjected to LC-MS/MS and data analysis.

FIGURE1. Complete Workflow including Flash Digest, LC-MS and Data Analysis



Sequence coverage maps of both light chain and heavy chain of native, non-reduced mAb were generated from PepFinder software. Without reduction of disulfide linkages, a 30-min digestion time is adequate to achieve good sequence coverage of >83% for light chain and >79% for heavy chain, indicating an excellent digestion efficiency. The uncovered sequences on light (Figure 2) and heavy chains (not shown) are due to the non-reduced disulfide bonds on cysteine residue. As shown in Table 1, sequence coverage of light and heavy chains was not increased by extending digestion time to 120 min.

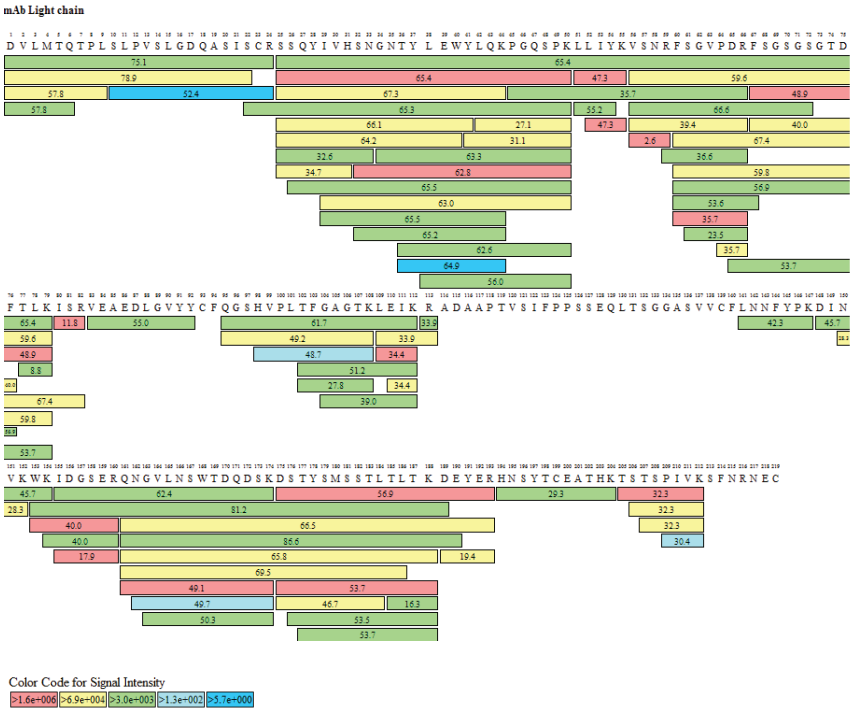
TABLE 1. Sequence Coverage Summary of Native, Non-reduced IgG at Various Digestion Times

Sequence Coverage	15min	30min	45min	60min	75min	90min	105min	120min
Light Chain	78.5%	83.6%	83.6%	83.6%	82.6%	83.6%	83.6%	83.6%
Heavy Chain	79.1%	79.1%	79.1%	79.1%	79.1%	79.1%	79.1%	79.1%

TABLE 2. Selected PTMs of Native, Non-reduced IgG at Various Digestion Times

Relative Abundance	15min	30min	45min	60min	75min	90min	105min	120min
N33+Deamidation (Light Chain)	27.12%	28.12%	28.90%	27.87%	26.96%	29.84%	29.85%	29.85%
N162+Deamidatio n (Light Chain)	15.98%	17.23%	18.38%	19.29%	20.29%	21.39%	21.68%	22.16%
M180+Oxidation (Light Chain)	0.26%	0.35%	0.49%	0.70%	0.97%	0.98%	1.01%	1.31%
N83+Deamidation (Heavy Chain)	1.44%	1.44%	1.62%	1.79%	1.81%	2.13%	2.14%	2.12%

FIGURE 2. Sequence Coverage Map (83.6%) of Native, Non-reduced IgG Light Chain



Sequence Characterization of IgG mAb

When the same IgG is further reduced and alkylated, sequence coverage of both IgG light chain (Figure 3) and heavy chain are 100% and 97.1%, respectively. On the sequence coverage map (Figures 2 and 3), most peptide sequences are mapped multiple times indicating by different color bars below the sequence, which greatly increases the identification confidence. The color bar represents signal intensity of the identified peptide. The number within the bar is the retention time of eluting peptide.

This data is comparable to the result generated by an overnight digestion protocol in which IgG was denatured by guanidine, reduced by DTT and alkylated by IAA, followed by trypsin digestion overnight (data not shown). The sample preparation efficiency is significantly improved with Flash Digest kit.

Simultaneous Identification and Relative Quantitation of PTMs in Oxidatively Stressed IgG Samples

Differential oxidative stress was induced by hydrogen peroxide at 15, 30, 60, 90, 120 mins. After simple trypsin digestion, samples were analyzed by LC-MS/MS. PTMs such as oxidation, deamidation, and glycation at different amino acid sites are identified with high confidence levels as shown in the summary (Table 3) from software.

The relative abundance of each modification in different samples under same LC-MS condition is calculated. The oxidative study of IgG shows that oxidation of methionine (M) 49, 304 and 393 in the heavy chain is dose-dependent as the oxidation reaction time increases from 30 to 120 min, while unquenched sample demonstrated significantly higher percentage of oxidation. M49 oxidation was identified by isotopic mass and confirmed by MS/MS spectrum in PepFinder software. The experimental MS/MS spectrum was annotated automatically in the software. The well matched predicted (top panel) and experimental (bottom panel) spectra are shown in Figure 4, demonstrating the high confident identification and confirmation of peptide with oxidation modification.

Relative percentages of each glycoform, double oxidation of methionine and deamidation of asparagine (N) , H_2O loss of threonine, serine, aspartic acid and glutamic acid, glycation on lysine did not change over the reaction time as expected. Double and triple oxidation of cysteine were monitored but not observed.

FIGURE 3. Sequence Coverage Map (100%) of Digested, Reduced and Alkylated IgG Light Chain

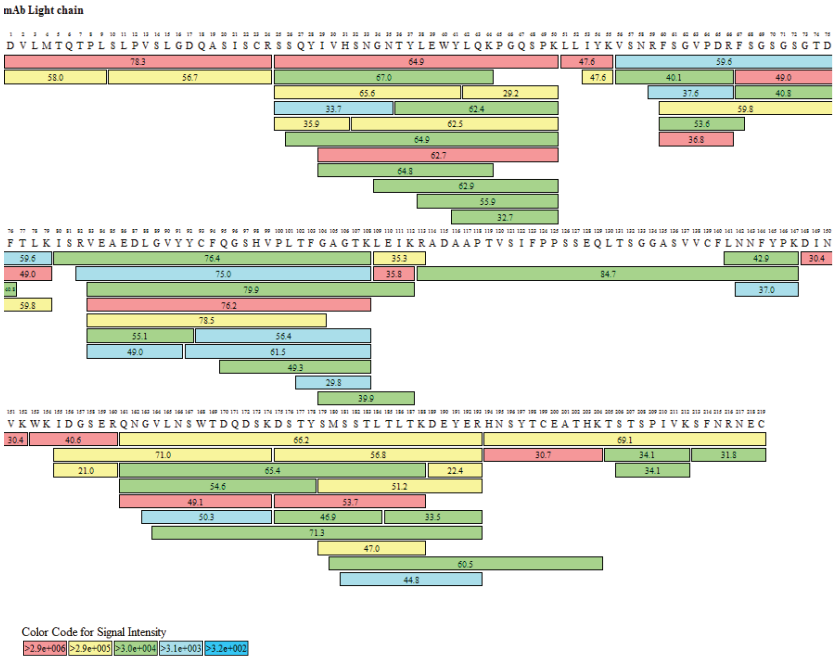
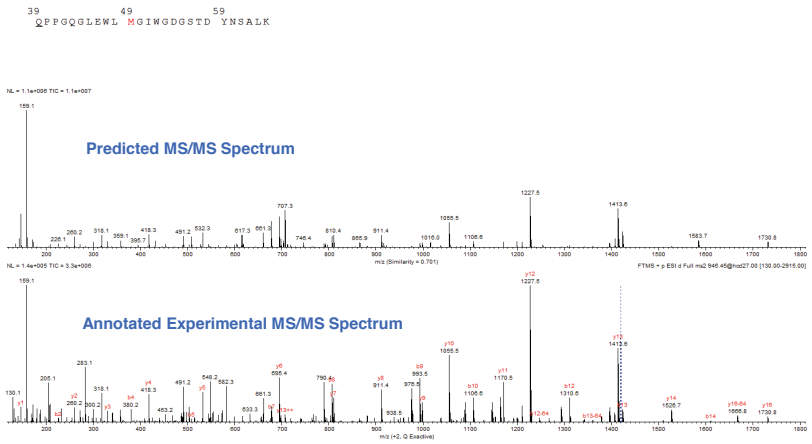


TABLE 3. Summary of Selected Identified PTMs and Major Glycoforms of Oxidatively Stressed IgG Heavy Chain

Relative Abundance	15min	30min	60min	90min	120min	Unquenched
M49+Oxidation	9.67%	9.51%	10.70%	10.84%	11.43%	74.30%
~M49+Double Oxidation	0.12%	0.13%	0.15%	0.13%	0.15%	0.12%
N60+Deamidation	4.34%	5.14%	4.95%	5.55%	5.25%	4.86%
M304+Oxidation	15.35%	18.50%	24.20%	30.35%	36.76%	86.69%
M393+Oxidation	13.99%	14.28%	18.53%	21.67%	25.80%	84.36%
N292+A1G0F	15.48%	16.51%	16.17%	16.86%	16.00%	16.91%
N292+A1G1F	4.04%	4.87%	5.24%	5.17%	4.99%	4.94%
N292+A2G0F	34.82%	34.35%	35.59%	33.71%	35.34%	33.60%
N292+A2G1F	36.86%	36.12%	34.54%	36.53%	34.89%	36.36%
N292+A2G2F	7.57%	7.82%	7.62%	7.11%	7.99%	7.28%

FIGURE 4. MS/MS Spectra of Triply Charged Peptide with Methionine Oxidation



Conclusion

A complete workflow has been developed for the fast and comprehensive sequence and post-translational modifications analysis of monoclonal antibodies.

- A 30-min digestion time demonstrated sufficient digestion efficiency of immobilized trypsin column for IgG mAb. Good sequence coverage of native, non-reduced IgG light and heavy chains were obtained. Further reduction and alkylation increased sequence coverage to 100% and 97% for light and heavy chains, respectively.
- Oxidative study results show that oxidation of methionine 49, 304 and 393 in IgG heavy chain is dose-dependent as the oxidation reaction time. However, major glycoforms did not change as expected.
- This workflow could greatly shorten the sample preparation and data analysis time while providing great sensitivity to detect low level PTMs. Additionally, any unintentionally incurred oxidative stresses during biopharmaceutical production may be rapidly analyzed for impact on production.

Reference

1. Samaranayake H, Wirth T, Schenkwein D, Raty JK, Yla-Herttuala S (2009) Challenges in monoclonal antibody-based therapies. *Ann Med* 41: 322–331.
2. Durocher Y, Butler M (2009) Expression systems for therapeutic glycoprotein production. *Curr Opin Biotechnol* 20: 700–707.

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