Top-down Characterization of Monoclonal Antibody on an Orbitrap Fusion Lumos™ Tribrid Mass Spectrometer

Seema Sharma, Stephane Houel, Christopher Mullen, Chad Weisbrod, Romain Huguet, John Syka, Dave Horn, Jonathan Josephs, Jae Schwartz, Vlad Zabrouskov
Thermo Fisher Scientific, San Jose, CA

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ABSTRACT

Mass analysis and top-down characterization of a monoclonal antibody was conducted on a modified Orbitrap Fusion Lumos mass spectrometer. The mass spectrometer was fitted with a 213 nm wave-length Nd:YAG diode pumped solid state laser to allow for UVPD fragmentation in the linear trap. Multiple fragmentation modes were investigated and instrument parameters that influence the kinetics and extent of fragmentation were monitored with a goal to optimize sequence coverage.

INTRODUCTION

Intact mass analysis is frequently used for the screening of therapeutic monoclonal antibodies. More recently, top down analysis of the antibody sub-units has gained interest as a means of sequence validation and the assignment of glycan modifications or drug attachment site. In this work, we conduct mass analysis of the intact monoclonal antibody (mAb), as well as top down characterization of the enzymatic digests (LC, Fc/2, Fd sub-units) and the reduced heavy chain product. Wide isolation windows were used to target multiple charge states of the eluting proteins to allow for a precursor AGC target of 1e6 to be achieved within injection times suitable for the LC-MS time scale. Critical parameters such as the precursor-reagent reaction times for ETD HD and the number of laser pulses for UVPD were investigated for the top-down fragmentation experiments.

MATERIALS AND METHODS

Sample Preparation and Liquid Chromatography

The SILuLite antibody standard was purchased from Sigma Aldrich and IdeS protease was purchased from Promega corporation. The intact mAb was deglycosylated using PNGase F, denatured using 8M Gu-HCl and reduced with 100mM DTT to produce the heavy chain. The monoclonal antibody was diluted in Tris HCl 0.1M and digested with the IdeS protease prior to denaturing and reduction for the sub-unit analysis. A Thermo Scientific™ UltiMate™ 3000 RSLC system operating at 400ul/min was used for the RP-LC analysis. A Thermo Scientific™ MabPac RP column (2.1 x 100 mm) was used for all the analyses and the column compartment temperature was maintained at 80C. The samples were desalted online and the flow was diverted to waste during this time. 1ug of sample was loaded for the intact mAb mass analysis and 5ug was loaded for the top-down experiments. A 8ug sample was loaded for the heavy chain isotopic mass analysis. The intact mAb was analyzed using an organic gradient of 30% - 45% Acetonitrile in 0.1% Formic acid over 5 minutes. The IdeS sub-units were separated using an organic gradient 20% - 35% over 29 minutes. A gradient of 20-45% organic solvent in 29 minutes was used for the heavy chain analyses.

Data Processing

BioPharma Finder 1.0 SP1 was used to deconvolute the intact mAb spectrum using the ReSpect algorithm (sliding window) and Xtract algorithm was used for deconvoluting the isotopically resolved data. The Xtract deconvolution parameters were set as follows- fit factor 80%, remainder threshold 25%, and signal to noise threshold 1.5 or higher. The resulting list of monoisotopic masses was searched using ProSight Lite with a fragment mass tolerance of 10ppm. The sequence maps for the combined fragmentation results were generated in ProSight Lite by combining the matched *c/z* ions from the ETD experiments, *b/y* ions from the CID experiments, and *a/x/b/yc/z* ions from the UVPD experiments.

Mass Spectrometry

The Ion funnel RF value was set at 60% for the intact mAb mass analysis and at 30% for all other experiments. The ion transfer tube temperature was set at 400C and vaporizer temperature was set at 200C for the intact mAb analysis. The ion transfer tube and vaporizer temperatures for all other experiments were set at 300C and 100C respectively. An AGC target of 2e5 was used for intact mass analysis and AGC target 1e6 was used for top-down analysis. A 300 *m/z* wide isolation window centered at *m/z* 1000 was used for the top-down analyses. MS data for the intact mAb was acquired at a high mass range with Orbitrap resolution setting 15K and using 10 microscans. For all other experiments, the instrument was operated in intact protein mode with an IRM pressure setting of 1mtorr and Orbitrap resolution 240K. Data was acquired using 5 microscans for the analysis of the antibody subunits and the heavy chain. Reagent AGC target was set at 7e5 and reaction times 3ms- 25ms were monitored for the ETD HD experiments. The instrument was equipped with a 213 nm UV laser (Nd:YAG diode pumped solid state laser). The laser energy was set at 2.5 uJ per pulse and the number of pulses was varied from 10-30 for the UVPD fragmentation experiments. The CID collision energy value was previously optimized at 25% and the normalized HCD energy values 7- 15% were monitored.

RESULTS

Figure 1. LC-MS analysis of Intact mAb. Mass spectrum of the mAb acquired by averaging over the LC-MS peak. The inset is a zoom in for charge state 52+ showing base line resolution for the major glycoforms and their mass measurement accuracies.

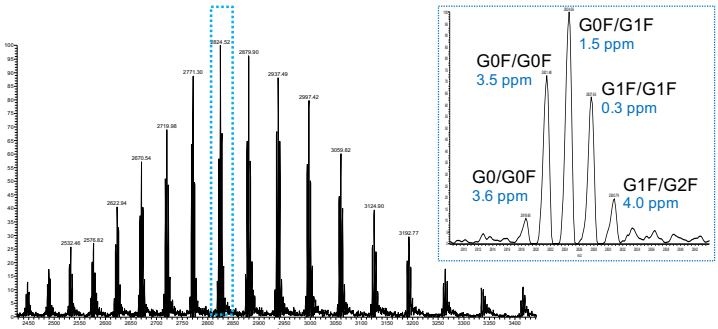

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Figure 2. LC-MS ETD HD analysis of mAb sub-units. Total ion chromatogram, mass spectra and protein sequence coverage (*c/z* ions) for the Fc/2, light chain and Fd sub-units analyzed using ETD HD fragmentation using 5 ms precursor-reagent reaction time.

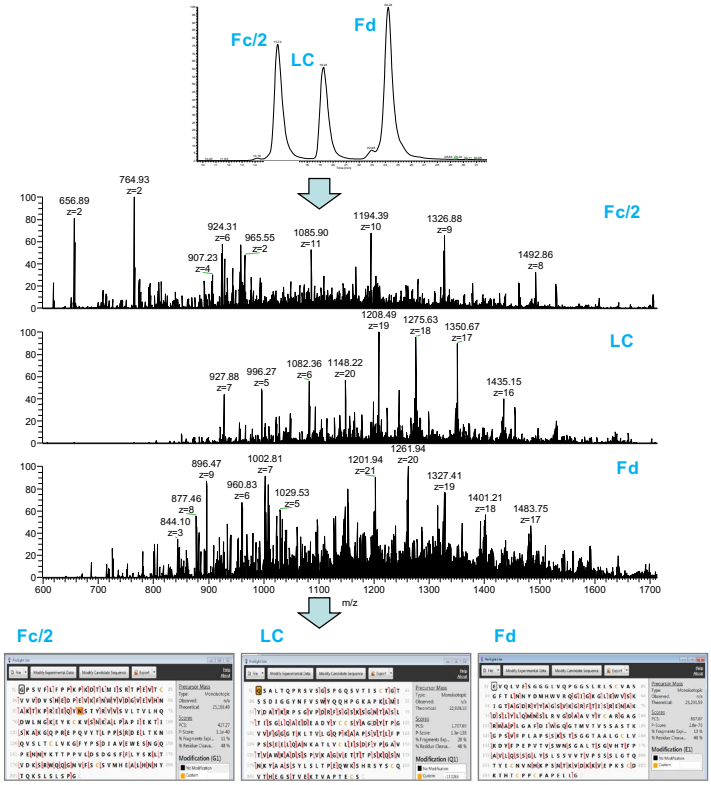


Figure 3. LC-MS UVPD analysis. UVPD fragmentation spectrum for the light chain at A) 20 UVPD pulses and B) 25 UVPD pulses. The insets show the protein sequence coverage. Many unique fragments can be identified from the fragmentation spectra acquired using different number of pulses.

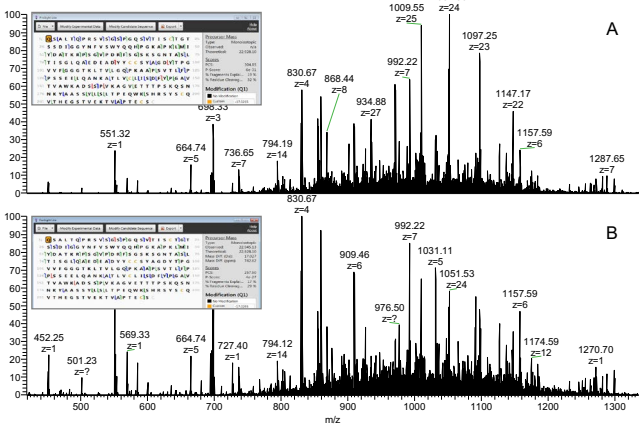


Figure 4. UVPD fragmentation using different number of pulses. 40% sequence coverage for the light chain was obtained by combining the results from fragmentation spectra with number of pulses 20 and 25.

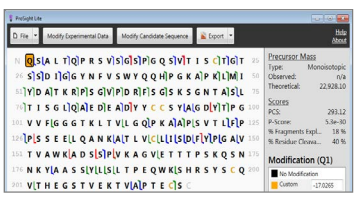


Figure 5. Frequency of fragment ion masses detected at different ETD HD reaction times. Results for Fc/2 show higher frequency for the larger fragment ions at shorter precursor-reagent reaction times.

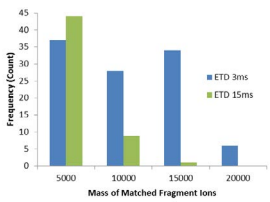


Figure 6. ETD HD spectrum at varying precursor-reagent reaction times. Comprehensive sequence coverage can be obtained by combining fragmentation results at different ETD precursor-reagent reaction times. A) LC-MS ETD HD spectrum of the Fc/2 sub-unit at reaction time 3ms B) LC-MS ETD HD spectrum of the Fc sub-unit at reaction time 15ms.

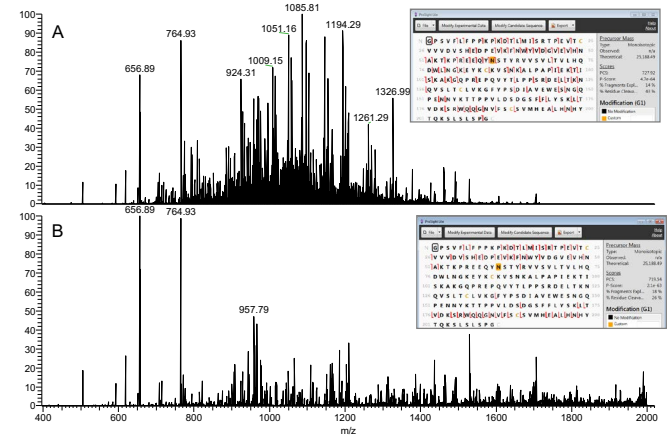


Figure 7. Sequence coverage for antibody subunits. A) Sequence coverage obtained for the antibody sub-units from different top-down fragmentation LC-MS experiments. B) Unique fragments identified from the UVPD top-down LC-MS experiments contribute towards increasing the sequence coverage for the antibody sub-units.

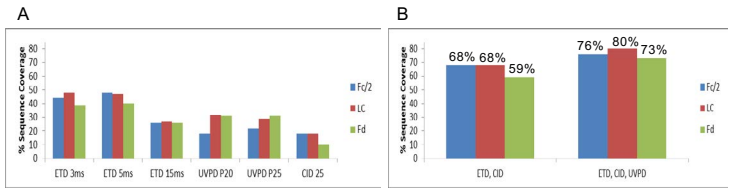


Figure 8. Sequence maps for monoclonal antibody sub-units. Comprehensive sequence coverage obtained by combining fragmentation results from ETD HD at reaction times 3ms, 5ms, 15ms; UVPD at number of pulses 20, 25; and CID 25%. Sequence coverage obtained for the Fc/2, light chain, and Fd was 76%, 80%, and 73% respectively.

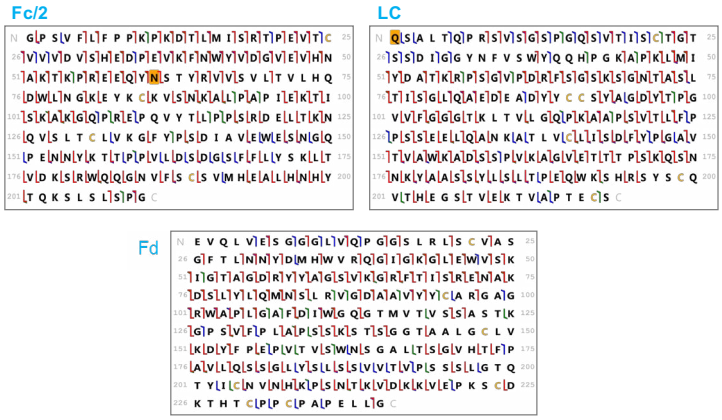


Figure 9. LC-MS high resolution heavy chain mass spectrum. Isotopic resolution of the deglycosylated heavy chain on LC-MS time scale at Orbitrap resolution setting 240K. A) Total ion chromatogram of reduced monoclonal antibody showing peaks corresponding to the light and heavy chains B) Heavy chain charge state envelope C) Zoom in of charge state 52+ showing isotopic resolution. The deconvoluted monoisotopic mass is within 2ppm mass measurement accuracy with the predicted deglycosylated heavy chain sequence with 2 deamidations .

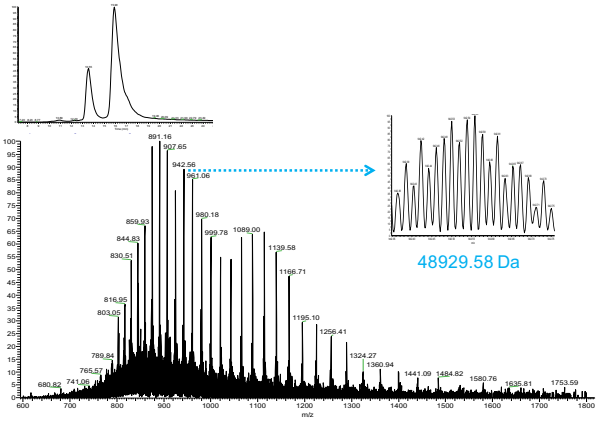
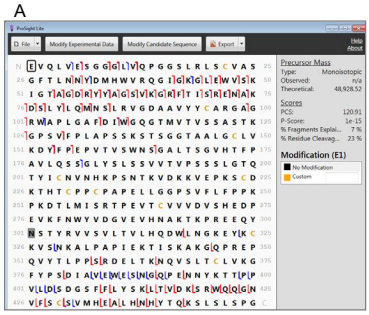


Figure 10. Top-down characterization of deglycosylated heavy chain. A) Sequence coverage (23%) obtained for the deglycosylated heavy chain by combining ETD HD fragmentation results at reaction times 5ms and 10ms with CID fragmentation results at collision energy 25%, B) Sequence coverage (24%) obtained from UVPD experiments with number of pulses 10, 15, 25, C) Combined sequence coverage 40% was obtained by combining the results from the ETD HD and CID experiments with that obtained from the UVPD fragmentation results.

A



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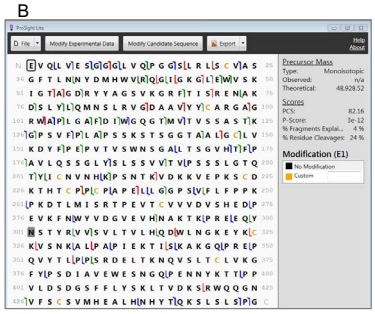

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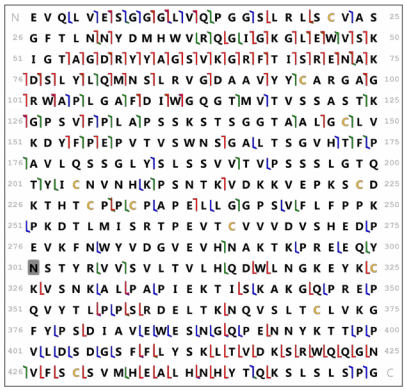

CONCLUSIONS

LC-MS analysis was performed on the intact monoclonal antibody, the IdeS sub-units and the reduced heavy chain product.

ETD HD, UVPD and CID top-down fragmentation modes were applied to obtain comprehensive LC-MS based sequence coverage 76%, 80% and 73% for the Fc/2, light chain and Fd sub-units respectively.

Isotopically resolved LC-MS spectrum for the deglycosylated heavy chain was obtained at an Orbitrap resolution setting 240K.

ETD HD, CID and UVPD top-down fragmentation results were combined to achieve 40% sequence coverage for the deglycosylated heavy chain from 6 LC/MS runs.

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