Optimizing Top Down Analysis of Proteins on Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer

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Overview

Purpose: In this work we optimize top-down analysis of intact proteins with the goal of maximizing sequence coverage on a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer[1]

Methods: Intact proteins were characterized using ETD High Dynamic range (ETD HD)[2], HCD, and CID either by direct infusion into the mass spectrometer or online LC-MS

Results: We demonstrate high sequence coverage of intact proteins using top-down fragmentation on an Orbitrap Fusion Lumos Mass Spectrometer

Introduction

Top-down analysis allows the measurement of intact protein masses and provides information on post-translational modifications, proteoforms as well as the protein sequence via fragmentation of the intact proteins in the mass spectrometer. In this work, we present top down analysis of intact proteins on the Orbitrap Fusion Lumos mass spectrometer, equipped with the higher capacity ETD HD fragmentation mode and benefitting from improved transmission of ions into the Orbitrap analyzer afforded by Advanced Vacuum Technology. Results for intact protein fragmentation are presented for the standard proteins Ubiquitin, Carbonic anhydrase, Enolase and IgG. We also present top-down analysis of the extra cellular domain of TROP2, a transmembrane glycoprotein that is highly expressed in epithelial cancers. TROP2's function is regulated by intramembrane cleavage. Aberrant cleavage may drive tumorigenesis as accumulation of the intracellular domain in the nucleus can drive proliferation, transformation and self-renewal.

Methods

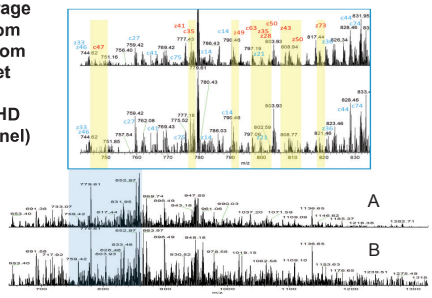
Sample Preparation and Liquid Chromatography

Protein standards (Ubiquitin, Carbonic anhydrase and Enolase) were purchased from Sigma Aldrich. Proteins were infused at 1pmol/ul at a flow rate 5ul/min for direct infusion experiments. Recombinant Human TROP2 protein (extracellular domain) was purchased from Sino Biological Inc. Intact IgG mass check standard and Trop2 were reduced and denatured with TCEP at 60C for 1 hour followed by PNGase incubation at 37C for deglycosylation. Concentrations of proteins on column were 2 pmol for Carbonic anhydrase, 4 pmol for Enolase, 100 ng for IgG mass check standard and 1ug for recombinant Trop2. A Thermo Scientific™ UltiMate™ 3000 RSLCnano system operating in microflow mode was used for RP-LC analysis of 1ul of protein loaded onto a Thermo Scientific™ ProSwift™ monolithic capillary column (200 um x 25 cm). Proteins were eluted with a gradient 25-65% Acetonitrile in 0.1% Formic acid over 6 min at a flow rate of 10ul/min

Mass Spectrometry

Intact proteins were characterized using the higher capacity ETD HD fragmentation mode as well as HCD and CID under the improved vacuum conditions afforded by Advanced Vacuum Technology[3] on the Orbitrap Fusion Lumos. The instrument was operated in intact protein mode with an ion routing multipole pressure of 1 -2 mtorr. Fragmentation spectra were acquired at 240K for direct infusion experiments and 120K for online LC-MS experiments. Results from multiple experiments isolating different charge states, ETD reaction times and different HCD and CID collision energy values were combined in order to maximize the sequence coverage. Top-down data was processed using Thermo Scientific™ ProSightPC™ 3.0 sp1 software with 15 ppm mass tolerance for the fragment ion searches.

FIGURE 1. 76% sequence coverage for 1pmol Ubiquitin obtained from ETD HD (A) compared to 65% from ETD (B) fragmentation. The inset shows a zoom-in of the fragmentation spectra for ETD HD (top panel) and ETD (bottom panel) acquired at 120K, 1uscan.



Results

Enhanced ETD performance

Improved precursor storage capacity in the ETD HD mode leads to a larger fragment ion population thereby increasing the dynamic range of the ETD acquisition scan. Advanced Vacuum Technology improves the transmission of ions, particularly those having larger cross-section into the Orbitrap analyzer by allowing a higher pressure in the ion routing multipole while maintaining ultra low vacuum in the Orbitrap. Both the improvements result in significant increase in sequence coverage of intact proteins. Figure 2, shows higher sequence coverage for Carbonic anhydrase obtained in much shorter amount of time on the Orbitrap Fusion Lumos MS as compared to Orbitrap Fusion MS. Figure 3 shows increased sequence coverage obtained for IgG light chain (56%) using the higher capacity ETD HD as compared to ETD (48%). The AGC target value was set to 1e6 for precursor ions in the ETD HD mode on an Orbitrap Fusion Lumos MS and 3E5 for ETD on the Orbitrap Fusion. The reagent target was set at 7e5 in both cases.

FIGURE 2. Sequence coverage for Carbonic anhydrase as a function of acquisition time for Orbitrap Fusion MS vs. Orbitrap Fusion Lumos MS. Spectra were acquired by isolating and fragmenting a single charge state at 240K in direct infusion mode.

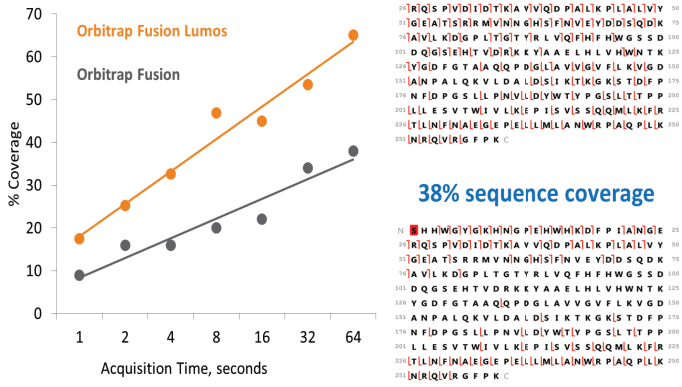


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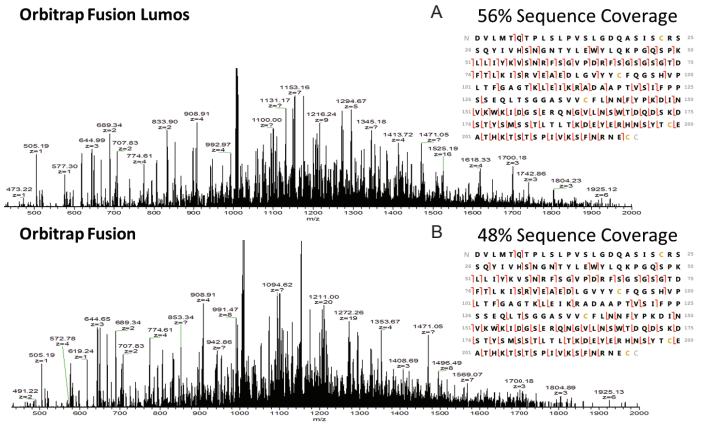
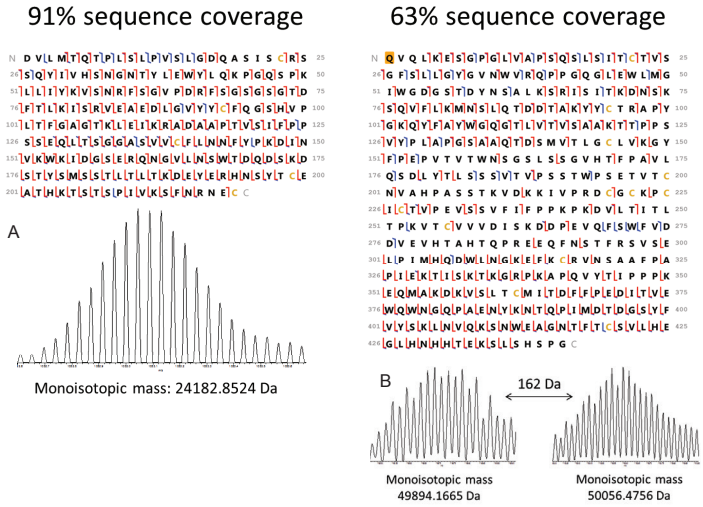
FIGURE 3. Comparison of the higher capacity ETD HD fragmentation of IgG light chain on the Orbitrap Fusion Lumos MS (A) with ETD fragmentation on the Orbitrap Fusion MS (B). MS/MS spectra were acquired using direct infusion by isolating and fragmenting charge state 24+ at 240K and averaging 500 micro scans. ETD reaction times were optimized to allow maximum sequence coverage.



Improved Top-down sequence coverage

The Orbitrap Fusion Lumos MS allows for increased gas pressure in the ion routing multiple while maintaining ultra-high vacuum in the Orbitrap region. This results in improved ion transfer and detection in the Orbitrap, especially when working with ions with large collisional cross-section, such as for large intact proteins. Figure 4 shows the high sequence coverage achieved for top-down analysis of IgG light and heavy chains using direct infusion. Different charge states were isolated in the mass selecting quadrupole with isolation window 5 *m/z* and fragmented using ETD HD with reaction times up to 15 ms, CID collision energy values 25% -55%, and HCD collision energy values from 8% -15%.

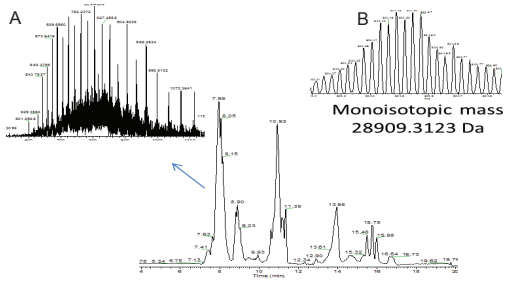
FIGURE 4. Isotopic resolution at 240K for IgG light (A) and heavy chains (B). 91% sequence coverage for the light chain and 63% sequence coverage for the deglycosylated heavy chain was obtained from top-down analysis. Results from isolation of different charge states and ETD HD reaction times, HCD and CID collision energy values were combined in order to generate the sequence maps.



Top-down LC-MS Analysis of Intact proteins

Top down analysis of intact proteins was performed using online LC-MS chromatography as described in the methods section. The ion routing multipole pressure was set at 2 mtorr and targeted MS/MS spectra were acquired at 120K Orbitrap resolution. Precursor isolation widths were set at 5 *m/z* for isolating single charge states and 200 *m/z* for co-isolating and fragmenting multiple charge states of the protein. 68% sequence coverage for Carbonic anhydrase and 41% sequence coverage for Enolase was obtained in the LC-MS experiments by combining results from ETD HD, CID and HCD (data not shown here).

FIGURE 5. Base peak chromatogram of deglycosylated recombinant Trop2 protein analyzed using online LC-MS at 15K Orbitrap resolution setting. Peak 1 corresponds to Trop2. The inset (A) shows the charge state distribution at 15K and (B) shows the isotopic resolution for charge state 36+ obtained at 240K Orbitrap resolution using SIM scan.



LC-MS based top-down analysis of Trop2 protein

The experimental mass measurement for the deglycosylated Trop2 recombinant protein suggests that the N-terminal truncation extends four amino acids further (1-30) than the predicted signal peptide sequence (1-26) for the extracellular domain. This is validated by sequence verification from the top-down fragmentation experiments (Figure 6). Furthermore, the fragmentation results also suggest modification of the N-terminal Glutamine to Pyroglutamate (Figure 7). Also shown in the sequence coverage data is evidence for deamidation of three Asparagine residues resulting from the separation of N-glycans upon reaction with PNGase F.

FIGURE 6. Top-down LC-MS analysis of Trop2. Fragmentation spectra are shown for ETD HD (A) reaction time 10 ms, HCD (B) collision energy value 10% and CID (C) collision energy value 45%. Wide isolation width for precursor selection was used to fragment multiple charge states over the LC-MS elution profile.

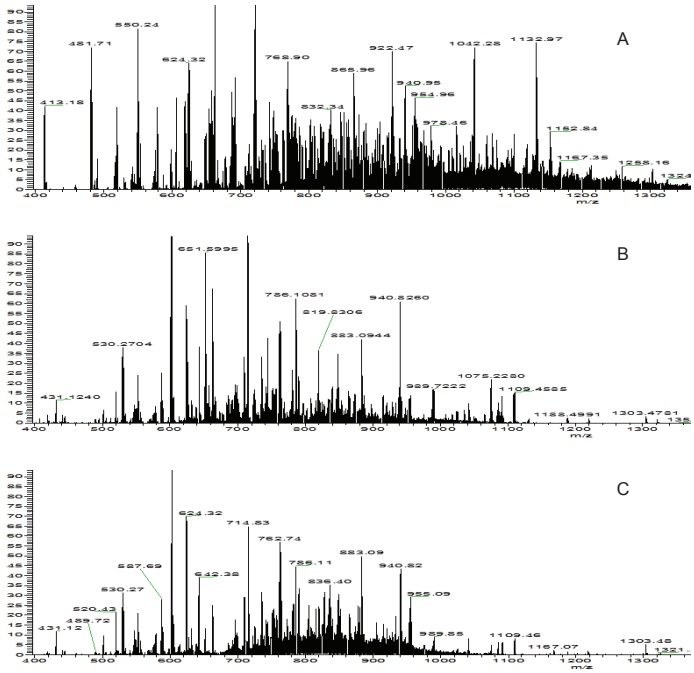


FIGURE 7. Sequence verification for Trop 2 protein via LC-MS top-down analysis. The fragmentation results indicate that the N-terminus truncation extends further (1-30) than the predicted signal peptide sequence (1-26) for the extracellular domain. This data also shows N-terminal Pyroglutamate conversion, as well as deamidation at three Asparagine residues following the reaction with PNGase F.

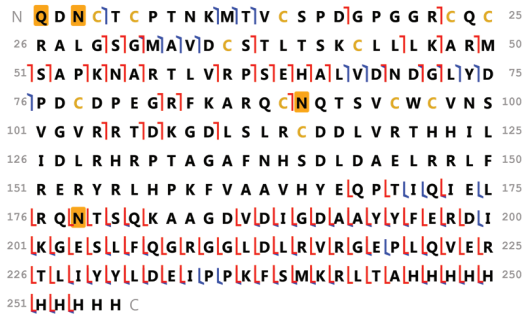
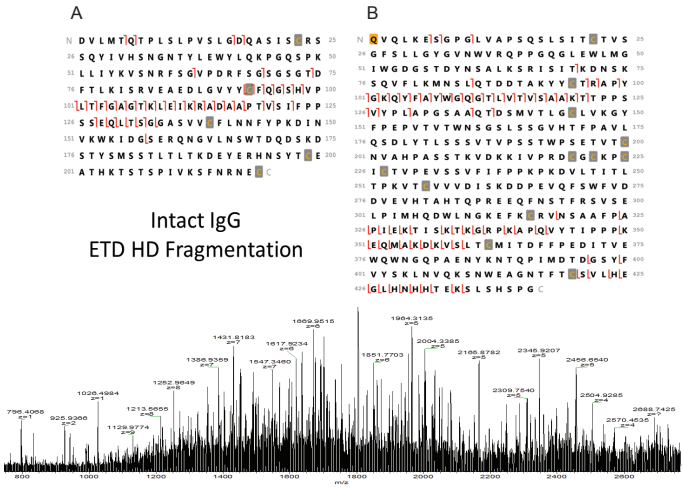


FIGURE 8. LC-MS based ETD HD analysis of intact IgG. A) 20% sequence coverage for light chain and B) 18% for the heavy chain was obtained based on LC-MS ETD HD analysis of intact IgG. LC MS/MS data was acquired at 120K Orbitrap resolution setting with 5 uscans. Results from ETD HD reaction times 10, 15, and 20 ms were combined.



Conclusion.

- The higher dynamic range ETD HD fragmentation mode provides increased sequence coverage at faster acquisition rates and time scales (increase from 38% to 65% for about 1 minute FT acquisition time demonstrated for Carbonic anhydrase).
- Results from top-down analysis via multiple fragmentation modes for reduced IgG were combined to achieve 91% sequence coverage for the light chain and 63% sequence coverage for the heavy chain.
- Top-down LC-MS analysis of Carbonic anhydrase and Enolase via multiple fragmentation modes provided 68% and 41% sequence coverage respectively.
- Trop 2 sequence verification by top-down analysis shows truncation of the N-terminal (1-30) that extends further than the predicted signal peptide sequence (1-26) for the extracellular domain. The results also indicate Pyroglutamte conversion at the N terminus and deamidation of three Asparagine residues following the reaction with PNGase F.
- We demonstrate 18% sequence coverage for IgG light chain and 20% sequence coverage for the heavy chain based on LC-MS ETD HD analysis of intact IgG.

References

1. Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer, Product Specifications
2. C. Mullen, et al., “Considerations for Attaining Improved ETD Performance for Top Down Applications,” Poster 48, Tuesday 6/2
3. J. Canterbury, et al., “Improvements for high-resolution analysis on a modified Tribrid mass spectrometer,” Poster 124, Tuesday 6/2