Improving Intact Protein and Top-Down Analysis by Orbitrap Mass Spectrometry

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Overview

Purpose: Modifications to a standard iontrap-FTMS hybrid instrument for improved intact protein and top-down analysis.

Methods: Improvement in Orbitrap vacuum and higher trapping efficiency by trapping in HCD cell.

Results: Higher S/N of intact protein signal and higher sequence coverage for top-down analyses.

Introduction

Intact protein and top-down analysis can be significantly improved by two relatively simple modifications:

a) reduction of gas pressure in the C-trap and therefore improved vacuum in the Orbitrap

b) trapping of ions in the HCD collision cell

Under standard conditions, nitrogen pressure in the C-trap is adjusted to achieve good trapping efficiencies for small molecules and peptides. Although nitrogen is pumped away by three turbomolecular pumps in the region of the ion optics from the C-trap to the Orbitrap, the gas load into the C-trap influences the final vacuum in the Orbitrap. Improvements in the Orbitrap vacuum results in longer transient lifetimes.

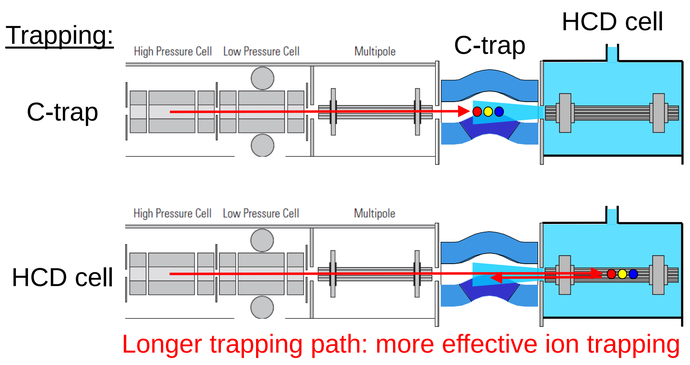

FIGURE 1. One nitrogen gas line going into the HCD collision cell serving as collision gas, nitrogen is leaking then into C-trap serving as cooling gas.

Methods

Sample Preparation

Humira® (adalimumab) [1]: The intact antibody (144 kDa) was dissolved in 0.1% FA to 1 µg/µL; 5 µg Humira® were loaded onto the column.

For analyzing Humira® light chain (24 kDa) and heavy chain (51 kDa) separately, 50 µg Humira® was reduced with DTT (20-fold molar excess, 56°C for 1 h) and alkylated with iodoacetamide (50-fold molar excess, room temperature for 30 min in the dark).

Instrument

A Thermo Scientific Surveyor MS Pump Plus was coupled to an Thermo Scientific Orbitrap Elite ETD hybrid mass spectrometer [2].

Samples were purified on a BioBasic-C4 column (150 x 1 mm, 5 µm particles (Thermo Fisher Scientific), solvent A: 0.1% FA, 2% ACN in H_2O, solvent B: 0.1% FA in ACN. The LC gradient was 7 min 20–40% B, 3 min 40–80% B at a flow rate of 100 µL/min.

The pressure in the C-trap was reduced via needle valve in the instrument. Changes in the instrument control software allowed trapping of ions in the HCD collision cell.

Data analysis was done using Protein Deconvolution 1.0 and ProSightPTM 2.0.

Results

Influence of Pressure and Trapping on Intact Antibody Mass Measurements

Proteins of the size of intact antibodies show only very short transient lifetimes due to their relatively big cross section. The method of choice for intact antibodies is to use the shortest transient duration (48 ms) available on the Orbitrap Elite hybrid MS. Figure 2 shows the transient of Humira® and the Fourier-transformation of the first 12 ms and the second part of the transient (ms 24-48). First 10 ms of the transient include most information of the intact protein.

For proteins of the size of intact antibodies the best trapping conditions are trapping in the HCD collision cell at high pressure. Trapping efficiency is almost doubled compared to the standard conditions.

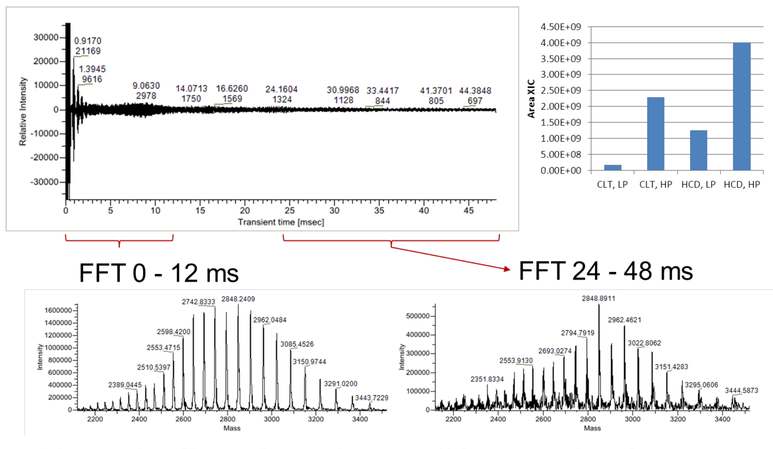

FIGURE 2. Effect of trapping conditions on signal intensities for intact antibody mass measurements shown with Humira®.

Influence of Pressure and Trapping on SIM Scans

For mass spectrometric analysis of intact proteins up to ~60 kDa, a SIM scan is the method of choice. Improvements in Orbitrap vacuum result in longer transient life times. Figure 2 shows transients and resulting spectra for SIM scans on a single charge state for carbonic anhydrase (29 kDa). The transients show typical “beats” of the protein signal.

Under improved Orbitrap vacuum settings, up to five beats survive in the transient whereas under standard pressure settings, only three beats survive. Trapping in the HCD cell shows improved trapping efficiency either. Spectra show a 4-fold improvement in S/N under optimized conditions compared to the standard conditions and higher absolute signal intensities.

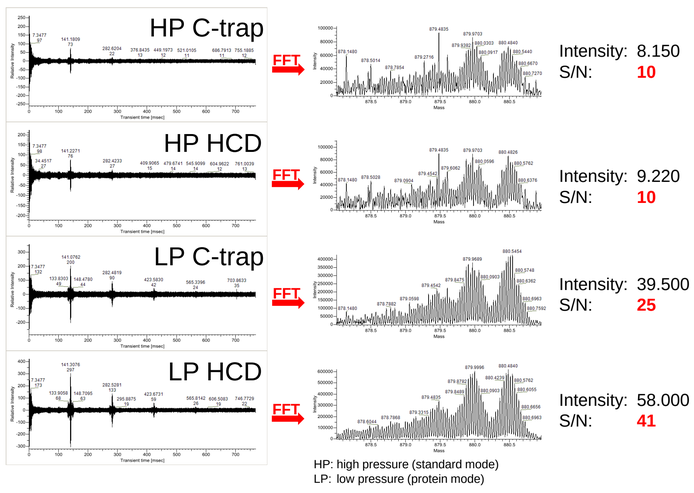

FIGURE 3. Effect of trapping conditions on signal intensities on example carbonic anhydrase, SIM on +33 charge state.

Figure 4 shows SIM scans of the heavy chain of Humira® under different pressure (HP and LP) and trapping conditions (HCD trapping and C-trap). Trapping in the HCD collision cell with improved Orbitrap vacuum settings shows the best spectrum quality, allowing even isotopically resolution of the heavy chain for precise deconvolution.

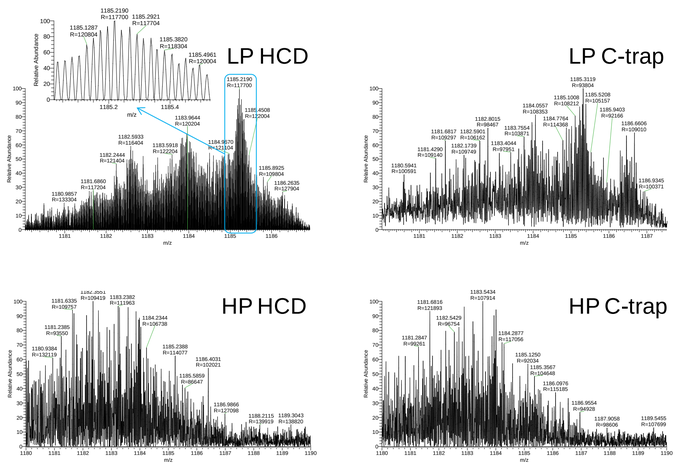

FIGURE 4. Humira® heavy chain SIM scans (z = 43) under different pressure and trapping conditions. Three scans (20 µS each) were averaged. Deconvoluted mass: Mr 50,891.13 Da.

Influence of Pressure and Trapping on MS/MS Scans

The final step in intact protein characterization is sequence verification and modification site determination (if any) via MS/MS.

The Orbitrap Elite ETD hybrid MS offers basically four fragmentation techniques which produce complementary sequence information: CID, HCD and ETD with precursor ion selection in the linear ion trap and *in-source* dissociation (SID) without precursor ion selection.

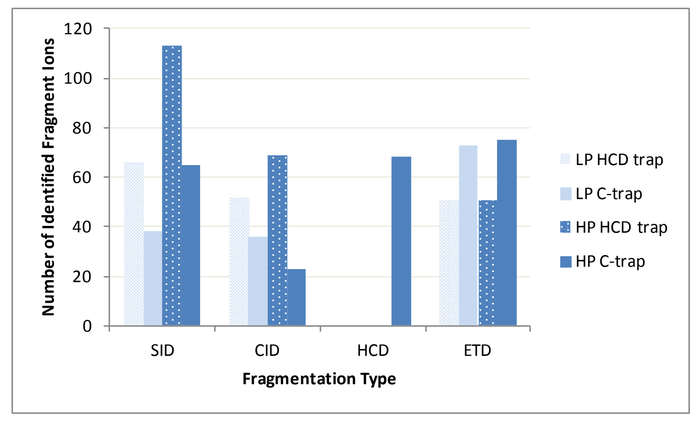

FIGURE 5. Humira® heavy chain (51 kDa): number of identified fragment ions using four fragmentation techniques under different trapping and pressure conditions. Ten scans (20 µS each) were averaged. HCD is only applicable to high pressure and C-trap trapping.

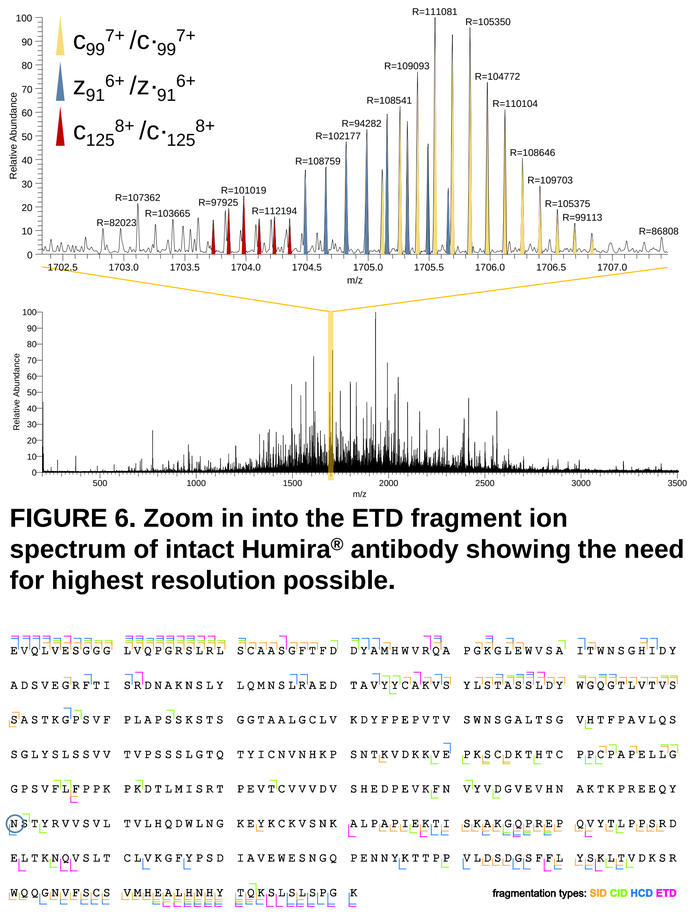

FIGURE 6. Zoom in into the ETD fragment ion spectrum of intact Humira® antibody showing the need for highest resolution possible.

FIGURE 7. Summarized sequence coverage of the Humira® heavy chain using fragmentation techniques SID, CID, HCD, and ETD. Optimized conditions: HCD trapping under high pressure settings. (N): Putative glycosylation position.

Conclusion

The results clearly show the advantages of trapping ions in the HCD collision cell for all modes for intact protein analysis with short and long transients as well as MS/MS scans.

We could further show that improved vacuum in the Orbitrap increases signal intensities for long transients.

Abbreviations

ACN, acetonitrile; CID, collision-induced dissociation; C-trap, curved linear trap; DTT, dithiothreitol; ETD, electron transfer dissociation; FA, formic acid; FFT, fast Fourier-transformation; HCD, higher energy collision-induced dissociation; HP, high pressure (delta pressure 0.3E-10 Torr); LC, liquid chromatography; low pressure LP (delta pressure 0.1E-10 Torr); µS, micro-scan; SID, *in-source* decay; SIM, single ion monitoring; XIC, extracted ion chromatogram.

References

1. Bondarenko P. V. *et al.*, *J Am Soc Mass Spectrom* **2009**, *20*, 1415–1424
2. Michalski A. *et al.*, *Mol Cell Proteomics* **2011**, *Epub ahead of print*

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