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

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Overview

Purpose: Modifications to a standard ion trap-Orbitrap™ hybrid instrument for improved intact protein and top-down analysis

Methods: Improvement in Orbitrap mass analyzer 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

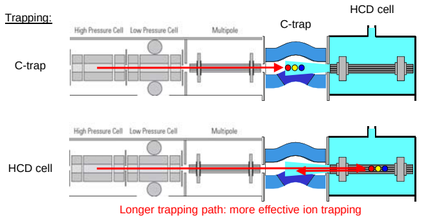
Intact protein and top-down analysis can be significantly improved by two relatively simple modifications:

a) Reduction of gas pressure in the C-trap and therefore improved vacuum in the Orbitrap mass analyzer

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 between the C-trap and the Orbitrap mass analyzer, the gas load into the C-trap influences the final vacuum in the Orbitrap mass analyzer (Figure 1). Improving the Orbitrap mass analyzer vacuum results in longer transient lifetimes required for the analysis of intact proteins.

FIGURE 1. A nitrogen gas line going into the HCD collision cell provides the collision gas. Nitrogen leaks into the C-trap and serves as the cooling gas.



Methods

Sample Preparation

Humira® (adalimumab) [1]: The intact antibody (148 kDa) was dissolved in 0.1% FA to 1 µg/µL; 5 µg of Humira were loaded onto the column.

For analyzing Humira light chain (24 kDa) and heavy chain (51 kDa) separately, 50 µg of 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).

Carbonic anhydrase was purchased from Sigma-Aldrich (C2522).

Liquid Chromatography

A Thermo Scientific Surveyor MS Pump Plus was coupled to a Thermo Scientific Orbitrap Elite hybrid ion trap-Orbitrap mass spectrometer equipped with electron transfer dissociation (ETD) [2].

Samples were purified on a Thermo Scientific BioBasic-C4 column (150 x 1 mm, 5 µm particles, 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.

Mass Spectrometry

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 Thermo Scientific Protein Deconvolution 1.0 and ProSight PC 2.0 software.

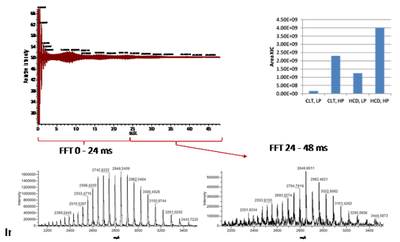
Results

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™ MS. Figure 2 shows the transient of Humira and the Fourier-transformations of the first 24 ms and the second 24 ms (ms 24-48). The first 20 ms of the transient includes most of the information on the intact protein.

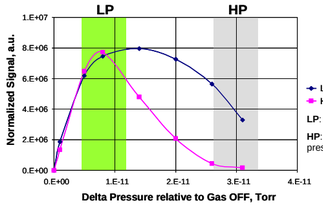
For proteins of the size of intact antibodies the best trapping conditions are trapping in the HCD collision cell at high pressure. Trapping efficiency is almost doubled compared to the standard conditions.

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



From Figure 3 it can be observed that for high-resolution, isotopically resolved carbonic anhydrase, delta pressure has to be reduced from ~3.0E-11 Torr (standard conditions) to ~1.0E-11 Torr relative to the background pressure.

FIGURE 3. Influence of pressure on signal intensity of carbonic anhydrase.



For LC timescale mass spectrometric analysis of intact proteins up to ~50 kDa, a SIM scan is the method of choice. Improvements in Orbitrap mass analyzer vacuum result in longer transient life times. Figure 4 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 mass analyzer vacuum settings, up to six beats survive in the transient, whereas under standard pressure settings, only two beats survive. Trapping in the HCD cell shows improved trapping efficiency. Spectra show more than a **25-fold improvement in S/N** under optimized conditions compared to the standard conditions and higher absolute signal intensities.

FIGURE 4. Effect of trapping conditions on signal intensities on example carbonic anhydrase, SIM on +34.

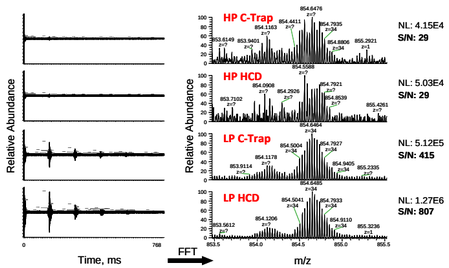
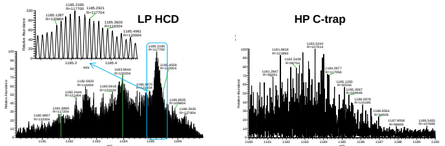


Figure 5 shows SIM scans of the heavy chain of Humira under optimized (LP and HCD trapping) and standard conditions (HP and C-trap). Trapping in the HCD collision cell with improved Orbitrap mass analyzer vacuum settings shows the best spectrum quality, allowing even isotopically resolution of the heavy chain for precise deconvolution.

FIGURE 5. Humira heavy chain SIM scans (z = 43) under different pressure and trapping conditions. 60 µscans 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 MS, when equipped with ETD, offers four fragmentation techniques that 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 6 shows the influence of pressure and trapping mode on the number of identified fragment ions for SID and ETD using carbonic anhydrase as a model protein. The source fragmentation energy for SID and the reaction time for ETD was gradually increased. The rationale for this was to start from low values to generate fragment ions at the lowest abundances in order to asses the influence of the pressure and trapping mode on the fragment ion transmission and detection.

FIGURE 6. Influence of the pressure and trapping mode on SID and ETD fragment ion detection on the example of carbonic anhydrase. Trapping in the HCD at low pressure is the most effective mode for SID and ETD.

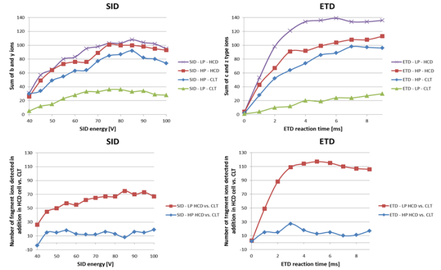


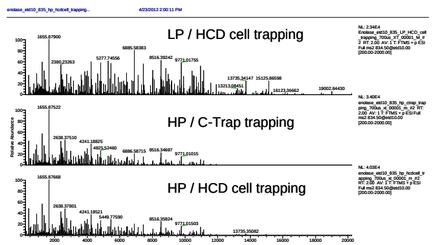
Table 1 shows the influence of the pressure and trapping mode on SID, CID, HCD and ETD on the example of the yeast enolase. As in the case of carbonic anhydrase, the LP HCD cell trapping was required to detect the largest number of fragment ions.

TABLE 1. Influence of the pressure and trapping mode on CID, HCD and ETD fragment ion detection on the example of yeast enolase.

	LP / HCD cell trapping	HP / C-Trap trapping	HP / HCD cell trapping	Number of µscans
etd10 < 10ppm	169	116	126	700
< 1.1Da	236	158	166	700
hcd18 < 10ppm	68	40		20
	107	76		80
cid20 < 10ppm	15	7	12	20
	47	24	33	100
sid80 < 10ppm	44	33	40	50
	57	39	46	90

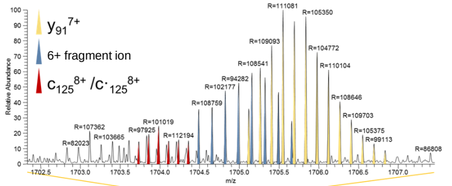
Figure 7 shows the advantage of using LP HCD cell trapping for detection of heavier ETD fragment ions.

Figure 7. Deconvoluted ETD spectra of yeast enolase for different pressure and trapping conditions.



These optimized conditions were applied to MS/MS analysis of an intact monoclonal antibody. Based on work done by Horn *et al.* [3], we decided to use 240k resolution required to resolve possible overlapping isotopic clusters. Figure 8 shows an ETD spectrum from a single LC run of intact Humira antibody highlighting the need of the highest resolution possible to resolve all fragment ions. Figure 9 displays the single LC run sequence coverage of the intact Humira antibody using ETD.

FIGURE 8. Expanded view of the ETD fragment ion spectrum of intact Humira antibody showing the need for highest resolution possible



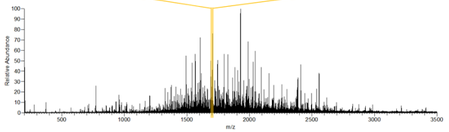


FIGURE 9. Single LC run sequence coverage of the intact Humira antibody using ETD. Optimized conditions: HCD trapping under low pressure settings.

Light chain:
36 c + 19 z fragment ions

Heavy chain:
40 c + 30 z fragment ions

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 showed as well that improved vacuum in the Orbitrap mass analyzer increases signal intensities for long transients.

Acknowledgements

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References

1. Bondarenko P. V. *et al.*, *J Am Soc Mass Spectrom* **2009**, *20*, 1415–1424.
2. Michalski A. *et al.*, *Mol Cell Proteomics* **2012**, *11*, O111.013698.
3. Horn *et al.*, *ASMS 2012 poster W180.*