

LC/MS Intact Mass Analysis of Immunoglobulin Gamma Antibodies by LTQ Orbitrap with ETD

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Overview

Purpose: Accurate mass LC/MS and top-down analysis of intact proteins, including monoclonal IgG antibodies, and CID and ETD MS/MS analysis with orbitrap detection.

Methods: LC/MS/MS analysis of proteins via Thermo Scientific LTQ Orbitrap. Direct infusion CID and ETD MS/MS of intact proteins with orbitrap detection. Data processing using Xtract Deconvolution, ProMass Deconvolution™, and ProSightPC™ v2.0.

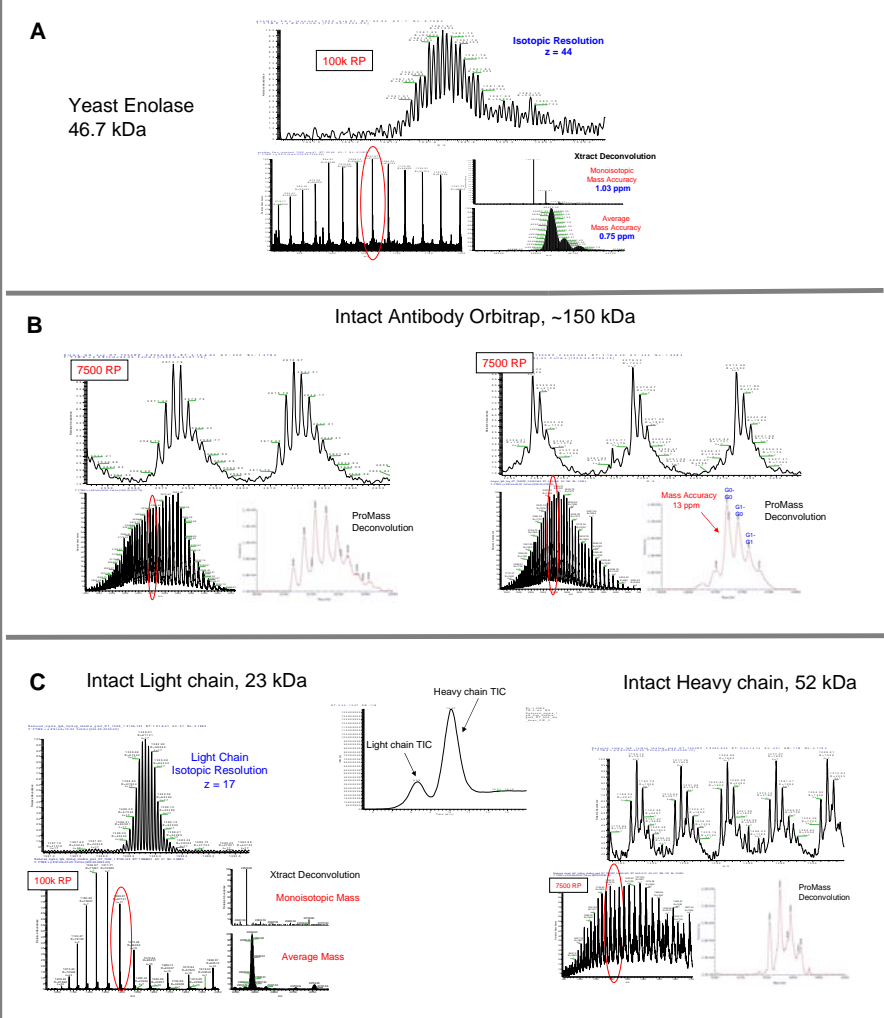
Results: Accurate mass LC/MS of intact and reduced antibodies achieved <15 ppm average mass for unresolved large proteins, <2 ppm on the monoisotopic mass (as determined by Xtract) for isotopically resolved yeast enolase (~47 kDa). Orbitrap CID-ETD MS/MS analysis of intact proteins, including intact and reduced antibodies, yeast enolase, with isotopic resolution of fragments and automated data interpretation with ProSightPC v2.0.

Introduction

High-throughput LC/MS/MS analysis of intact proteins affords great benefit for targeted characterization of the proteome, largely through preservation of sample integrity by avoiding disadvantages intrinsic to proteolytic digestion (e.g. less than complete coverage, induction of artificial modifications, loss of information on truncation state, etc.). In particular, the analysis of monoclonal antibodies is of great interest in biopharmaceutical development for the necessary characterization of these widely pursued therapeutic protein drugs. In addition to the strength of the orbitrap for peptide analysis, the orbitrap has most recently been demonstrated for analysis of intact proteins as large as ~150 kDa (intact antibodies) and for top-down MS/MS analysis of large intact proteins^{1,2}. This work extends the characterization to include ETD MS/MS of proteins with orbitrap detection, utilizing the high resolution achievable with orbitrap detection to explore sequence information at the intact level, with the added benefit of preserving labile modifications such as glycosylation with ETD for PTM analysis.

FIGURE 1. Analysis of Intact proteins with Orbitrap Detection.

A.) Isotopic resolution of intact yeast enolase (~46 kDa), acquired at 100,000 resolving power @ m/z 400 (100k RP). Mass accuracy of monoisotopic peak was measured as 1.03 ppm (as determined by Manual Xtract software). B.) LC/MS analysis of intact antibodies (150 kDa), demonstrating resolution of glycoforms. Mass accuracy was measured within <15 ppm (2 Da) for known sequences with ProMass Deconvolution, acquired at 7500 RP. C.) LC/MS Analysis of the reduced antibody, demonstrating isotopic resolution of light chain at 100k RP, and resolution of glycoforms of the heavy chain (7500 RP). Deconvolution with Xtract and ProMass Deconvolution. Reverse phase HPLC separation.



Methods

Samples: Intact proteins were purchased from Sigma™—yeast enolase, human IgG from plasma (sequence unknown). Monoclonal IgG antibody was provided by Amgen Inc (sequence known, not disclosed).

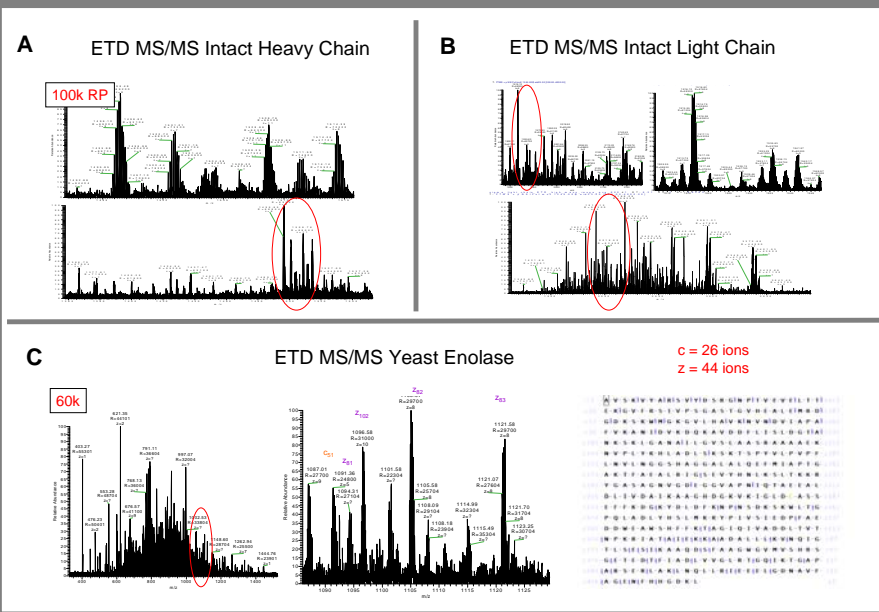
Direct Infusion: Pure protein samples were off-line desalted with Vivaspin® 500 MWCO spin columns (10K cutoff) or GE Healthcare NAP-5 Size Exclusion Columns (with Sephadex® G-25 medium), 2-3 rounds of buffer exchange into 10 mM ammonium acetate. Dilution into 50:50 Acetonitrile:Water + 0.1% formic acid, 2-20 pmol/μL protein. Direct infusion on a Thermo Scientific NSI or IonMax source into a Thermo Scientific LTQ Orbitrap XL ETD.

LC/MS analysis: Protein samples were on-line desalted/separated with reverse-phase chromatography on a protein microtrap (Michrom Bioresources, Inc.) with a Thermo Scientific Surveyor™ MS pump. 10 minute desalt or 30 minute separation method with gradient elution, either 20-80% B, or 30-80% B (A=0.1% formic acid, B = acetonitrile, 0.1% formic acid), 1-4 μg loads. LTQ Orbitrap XL ETD conditions—5 msec detect delay, high mass mode for intact antibodies, normal mode for all other proteins, elevated source voltages for antibody analysis (tube lens 230 V, source voltage at 50 V).

CID/ETD MS/MS: Isolation of 10-80 Da window to isolate multiple charge states of the same protein and/or to isolate heavy chain from light chain in gas phase for MS/MS. CID was used with 30% normalized collision energy. ETD was used with 5-23 msec activation depending on precursor charge, 5e5 fluoranthene target (signal at least 1e6 in profile), and full profile mode on (thresholding off).

FIGURE 2. ETD MS/MS Analysis of Intact proteins with Orbitrap detection.

A.) ETD MS/MS spectrum of Sigma antibody heavy chain with orbitrap detection, isolation 70 m/z, 4 msec ETD reaction time. B.) ETD MS/MS spectrum of Sigma light chain with orbitrap detection, isolation 10 m/z, 23 msec ETD reaction time. C.) ETD MS/MS spectrum of yeast enolase with orbitrap detection, isolation 80 m/z, 4 msec ETD reaction time, high mass mode. ProSightPC v2.0 assignment of fragments to sequence.



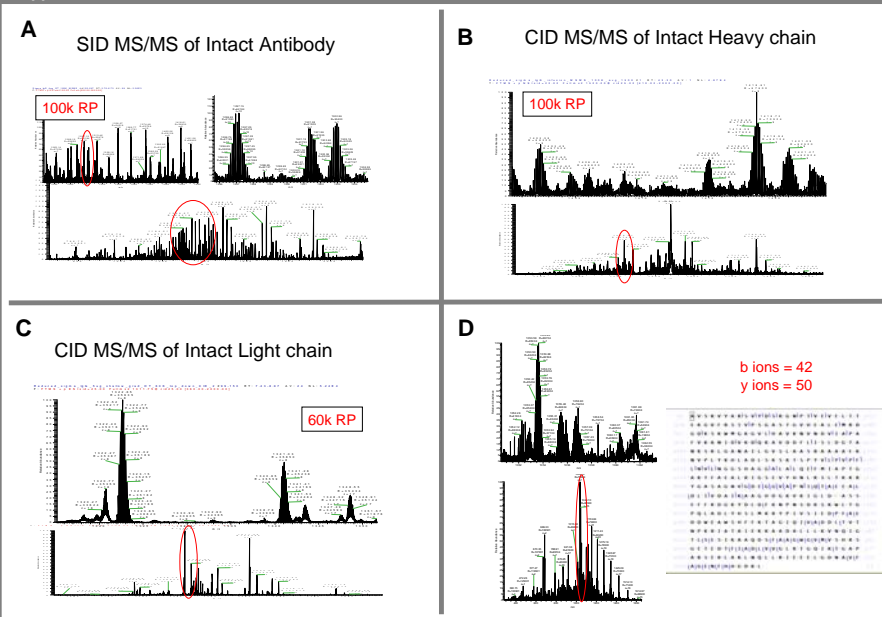
Results

Increasing the source voltages was necessary to best transmit higher m/z, and to assist in the removal of adducts. Increasing the resolving power of detection in the Orbitrap demonstrated little benefit for analysis of large proteins, such as intact antibodies, which could not be isotopically resolved. Therefore, it was determined that a resolving power of 7500 was optimal for their analysis. For proteins 46 kDa and below, a resolving power of 100K was used to achieve isotopic resolution and deconvolution to a monoisotopic mass with Xtract.

In general, a wider isolation window for pure protein CID or ETD is preferred as more charge states are fragmented together. Orbitrap ETD MS/MS of both heavy and light chains were achieved. Short activation times were preferred for ETD analysis of large proteins, on the order of 4-10 msec for enolase and heavy chain. Reaction times of ~20 msec were used for the light chain. Larger proteins required more averaging than smaller proteins, as the signal divides into more fragments with more isotopes. Turning full profile mode on (thresholding off) in the orbitrap mass spectrum produced more fragment coverage. Analysis with ProSightPC v2.0 produced automated sequence mapping of matching fragments. For the known sequence of enolase, CID and ETD fragmentation patterns were complementary, providing coverage in distinct regions of the protein.

FIGURE 3. LC/MS/MS Analysis of Intact proteins by CID with Orbitrap detection.

A.) LC/MS/MS fragmentation spectrum from Sigma intact antibody, utilizing source fragmentation (SID) at 100 V, acquisition at 100k RP. B.) CID MS/MS fragmentation spectrum from Sigma intact heavy chain with orbitrap detection, direct infusion, isolation in the gas phase, isolation 70 m/z. C.) LC/MS/MS CID fragmentation spectrum from Sigma intact light chain with orbitrap detection, isolation 30 m/z. D.) CID MS/MS spectrum of intact yeast enolase, 60k RP, isolation 20 m/z, and sequence coverage map from ProSightPC v2.0. Fragments <5ppm.



Conclusions

- Orbitrap detection of intact antibodies with <15 ppm mass accuracy on average masses of up to ~150 kDa was easily and routinely achieved with external calibration via LC/MS.
- Isotopic resolution of intact proteins/fragments as large as 46 kDa was achieved, facilitating unambiguous data interpretation and automated sequence mapping for full coverage of a protein sequence.
- CID MS/MS of intact proteins using Orbitrap detection was used to fragment intact antibodies with good isotopic resolution and to automatically assign the charge states of fragments for unambiguous characterization and sequencing of variable N-terminal regions of intact and reduced IgG antibodies.
- The fragmentation spectra of intact proteins from ETD MS/MS was generally more rich than CID, and the resolution of high charge state fragments with Orbitrap detection permitted N-terminal sequencing and retention of labile PTMs.
- Automated data processing with ProSightPC 2.0 was used for deconvolution to monoisotopic masses, fragment matching, identification and sequence mapping.

References

- Zhang, Z.; Shah, B. Characterization of variable regions of monoclonal antibodies by top-down mass spectrometry. *Anal. Chem.* **2007**, *79*, 5723-5729.
- Bondarenko, P. et al. Intact Mass and Top-Down HPLC/MS Analysis of Monoclonal IgG Antibodies on Orbitrap. 56th ASMS Conference on Mass Spectrometry, June 1-5, 2008.

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