APPLICATION BRIEF 73555

Characterization of IgG1 monoclonal antibody (mAb) oxidation variants at intact, subunit and peptide levels

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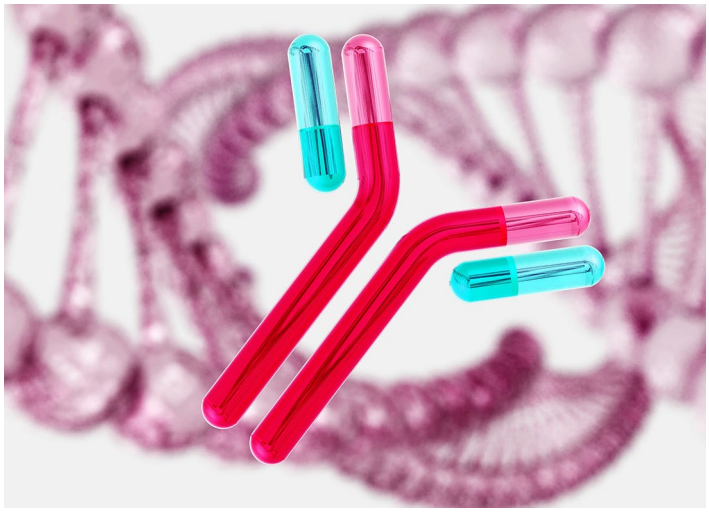

Key benefits

- Confident site-specific identification and localization of oxidation hotspots
- System versatility enables the analysis of protein biotherapeutics at the intact protein, subunit and peptide levels using standard conditions and simple set-up routines
- Exceptional spectral clarity enables robust deconvolution and simplified spectral interpretation

Goal

Demonstrate the utilization of the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer for the identification and localization of methionine oxidation of biotherapeutics via LC-MS analysis at the intact protein, subunit and peptide levels. The latest advances in high-resolution accurate-mass (HRAM) mass spectrometry can be deployed to pinpoint site-specific identification of oxidation hotspots in therapeutic proteins and detect with confidence low-level methionine oxidation through diagnostic MS/MS fragmentation at the peptide level.

Introduction

Oxidation is a common post-translational modification (PTM), with methionine, cysteine, histidine, and tryptophan residues particularly susceptible. During production of biotherapeutics, oxidation must be assessed and monitored because it can have an impact on the stability, safety and efficacy of the final drug product.

Here, we investigate the susceptibility of methionine to oxidation by subjecting the IgG1 monoclonal antibody ipilimumab to oxidative stress. Samples of ipilimumab were assessed at the intact protein, subunit and peptide level to pinpoint the locations of oxidation hotspots within the primary sequence. The sites of other potential critical quality attributes (CQA) are also determined using the Orbitrap Exploris 240 mass spectrometer.

Experimental

Liquid chromatography

- Thermo Scientific™ Vanquish™ Duo UHPLC system consisting of:
  - Two Vanquish Flex Binary Pumps (P/N VF-P10-A01) for tandem LC-MS operation
  - Split Sampler (P/N VF-A10-A-02)
  - Column Compartment (P/N: VH-C10-A-02)

Columns

- **Intact:** Thermo Scientific™ MAbPac™ RP column (P/N 088648), 4 µm, 2.1 × 50 mm, 5-min linear gradient
- **Subunit:** Thermo Scientific™ MAbPac™ RP column (P/N 088648), 2.1 × 50 mm, 4 µm, 16-min linear gradient
- **Peptide mapping:** Thermo Scientific™ Acclaim™ VANQUISH™ C18 column (P/N 074812-V), 2.2 µm, 2.1 × 250 mm, 45-min linear gradient

Solvents

A) Water with 0.1% formic acid (v/v), Optima™ LC/MS grade (P/N 10188164)

B) Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade (P/N 10118464).

0.3 mL/min. 10 µL injected for all three analyses

Mass spectrometer

- Orbitrap Exploris 240 mass spectrometer (P/N BRE725535) with Thermo Scientific™ BioPharma Option (P/N BRE725539)

MS acquisition

Application-specific MS tune and acquisition settings are templated and provided within the software. Settings are directly transferable from instrument to instrument, enabling easy method transfer and operational simplicity.

Software

- Thermo Scientific™ Xcalibur™ 4.2 software
- Thermo Scientific™ BioPharma Finder™ 4.0 software (P/N OPTON-30985) incorporating Sliding Window, ReSpect™, and Xtract algorithms

Automated proteolytic digestion

- Thermo Scientific™ SMART Digest™ Trypsin kits, Magnetic Bulk Resin option (P/N 60109-101-MB)
- Thermo Scientific™ KingFisher™ Duo Prime system (P/N 5400100)

Results and discussion

Ipilimumab - intact mass analysis

Ipilimumab was exposed to varying levels of hydrogen peroxide (H_2O_2: 50–500 ppm) for 24 hours to induce oxidation.

Ipilimumab – subunit mass analysis

Control and stressed samples of ipilimumab were digested using IdeS protease (FabRICATOR, Genovis), then denatured and reduced using guanidine hydrochloride and tris(2-carboxyethyl)phosphine (TCEP). The three subunits produced (Fc/2, LC and Fd') were then separated chromatographically.

Ipilimumab - peptide mapping

Control and forced degraded samples of ipilimumab were digested using the SMART Digest kits with automated sample handling on the KingFisher Duo Prime system. The resulting peptides were separated chromatographically.

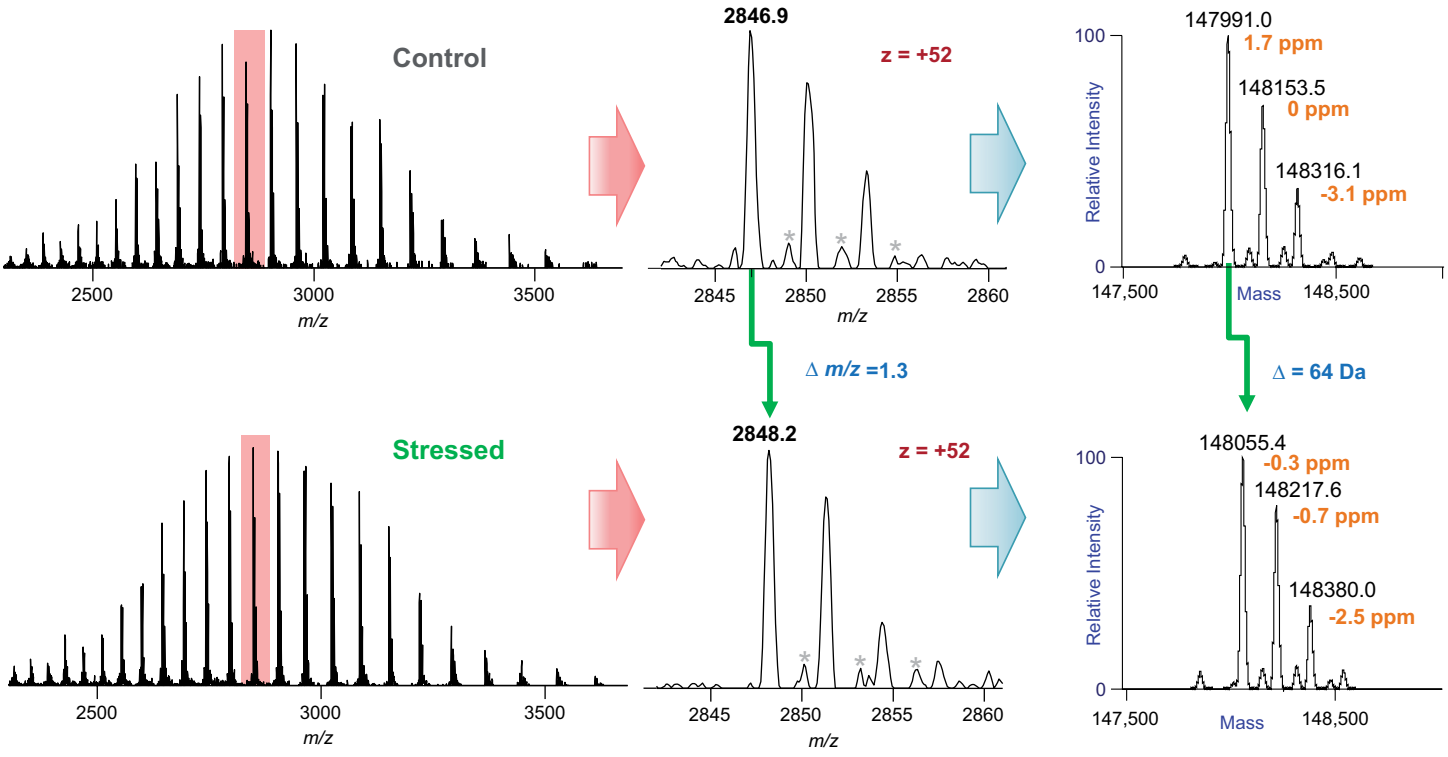

Figure 1. Oxidative stress at the intact protein level. The figure from left to right shows the full charge envelope of the intact mAb control and stressed samples (500 ppm H_2O_2), a zoom of the +52 charge state representing a baseline resolved glycoform pattern. The grey asterisks indicate solvent related adducts. Spectra on the right were obtained upon deconvolution using the ReSpect algorithm in BioPharma Finder software. Data were acquired with a resolution setting of 30,000 (at *m/z* 200), which provided average masses with mass accuracies below 4 ppm for the three most abundant glycoforms of ipilimumab. A mass shift of +64 Da was observed for the stressed ipilimumab sample, indicating potential oxidation at four methionine residues at the intact mAb level.

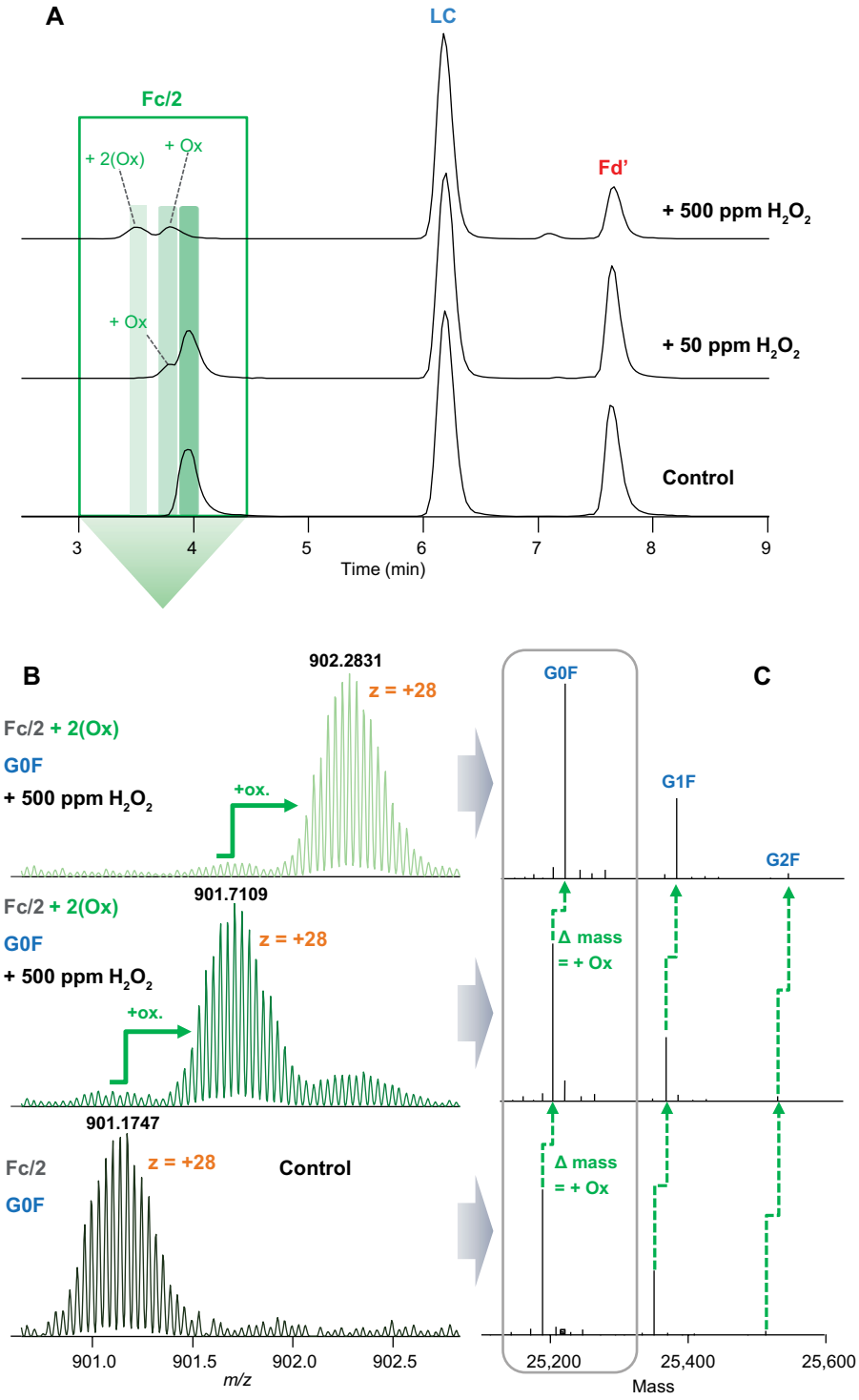

Figure 2. Oxidative stress detected at the subunit level. A) Zoom of base peak chromatograms (BPC) of separated G0F glycoform of the ipilimumab Fc/2 subunit obtained after IdeS digestion and reduction for the control and both stressed samples (50 and 500 ppm H_2O_2). B) Mass spectra of Fc/2 fragments acquired with a resolution setting of 120,000 (at *m/z* 200) showing near baseline-resolved isotope patterns of the +28 charge state of the Fc/2 G0F subunit, for the control sample plus the singly and doubly oxidized forms detected in the stressed sample (500 ppm H_2O_2). C) Results obtained upon deconvolution of the entire charge envelope including all Fc/2 subunit glycoforms using Sliding Window Xtract algorithm, obtaining accurate monoisotopic masses.

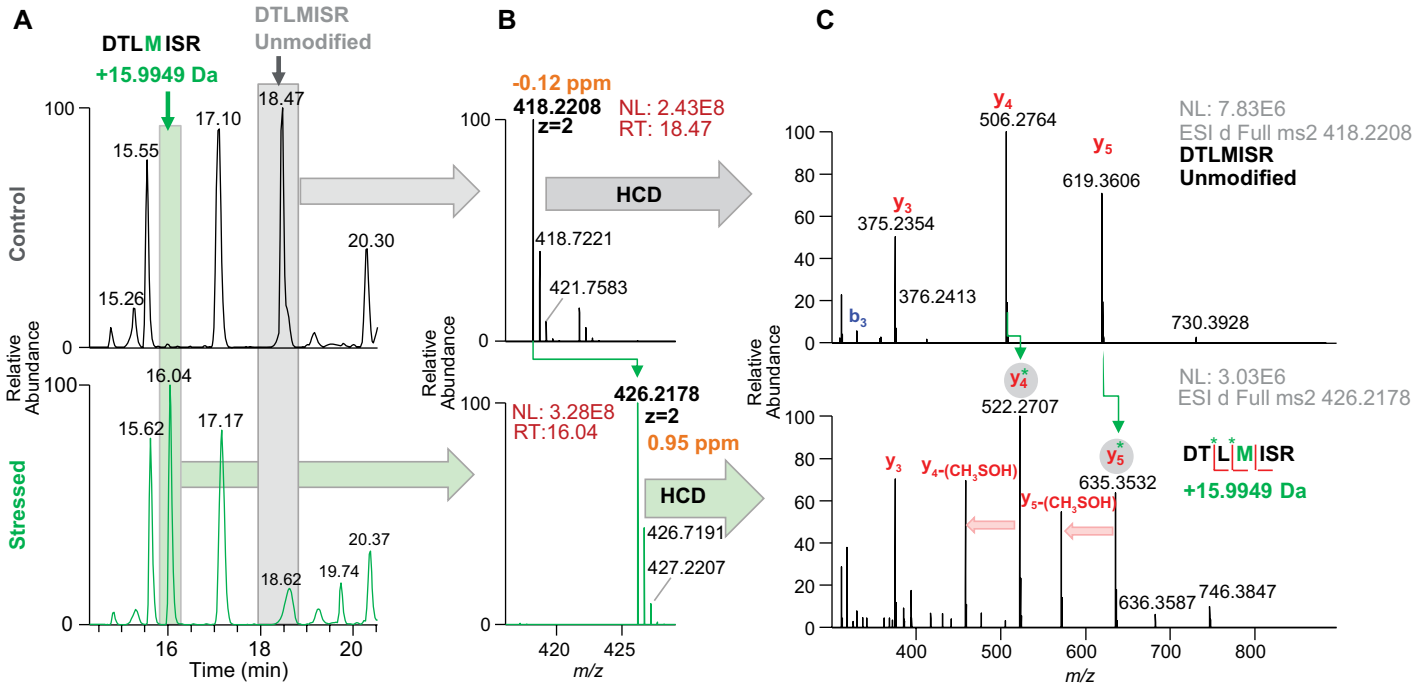

Figure 3. Oxidative stress detected at the peptide level. A) Peptide mapping total ion chromatogram (TIC) zoom of the control (upper) and stressed (lower) ipilimumab after digestion, specifically highlighting the tryptic 'DTLMISR' peptide of the heavy chain. A mass shift of +15.9949 Da, indicating methionine oxidation, was detected in the sample subjected to oxidative stress with 500 ppm H_2O_2. The modified variant is favoring earlier chromatographic elution at RT 16.04 min compared to the unmodified peptide eluting at 18.47 min. B) Zoom into the full MS spectra of DTLMISR displays the isotope patterns and relative abundances of the doubly charged peptides with <1 ppm mass accuracy, selected for HCD fragmentation. C) MS/MS fragment ion spectra for *m/z* 300–900. Peptide identification is further supported by confident assignment of a series of y-ions, with indicative shifts of +15.9949 Da observed for the y_4 and y_5 fragment ions of the oxidized peptide. Moreover, both the y_4 and y_5 fragment ions show the diagnostic neutral loss of methane sulfenic acid (CH_3SOH).

Conclusions

The Orbitrap Exploris 240 mass spectrometer delivers confident tracking of PTMs in mAbs at intact, subunit and peptide level with operational simplicity, simplified spectral interpretation and exceptional mass accuracy.

Acknowledgements

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Find out more at **thermofisher.com/OrbitrapExploris240**