Automated annotation of clipping related heterogeneities in Vedolizumab

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Introduction

For Biopharmaceuticals, degradation of the active drug substance during production, formulation or storage poses a threat to drug efficacy and safety and is a critical quality attribute (CQA). The detection of such truncated protein species -clipping variants- can be difficult to achieve through classic peptide mapping with trypsin. In this case study, a vedolizumab biosimilar was characterized after SpeB treatment, which cleaves IgG1 predominantly in the hinge region. Minor cleavage products with uncharacterized specificity have also been observed. We analyzed the reduced SpeB digest products with LC-MS using a high isotopic fidelity QTOF instrument to characterize the enzyme specificity and evaluate the performance of our clipping variants detection workflow. Middle-Down sequence validation measurements were subsequently performed to confirm the findings.

Methods

The Vedolizumab biosimilar sample (Polpharma Biologics) was measured after treatment with FabULOUS and IgGZERO enzyme kits (Genovis) followed by reduction using TCEP. Concentration after dilution was approximately 0.25 mg/ml. The sample was measured with a UHPLC system (Elute, Bruker Daltonics) coupled to a QTOF mass spectrometer (maXis II ETD, Bruker Daltonics). Injected sample amounts were 0.25 µg (intact mass) and 2 µg (Middle-Down experiment), using a BEH C4 column (Waters, 300Å 1.7 µm, 2.1 x 100 mm) and utilizing a 33.5 min gradient for mAb subunits and clipping products separation. Data were processed with Biopharma Compass 2021 software (Bruker Daltonics) to assign possible clipping variants to detected deconvoluted protein masses and afterwards to confirm sequences based on ETD and CID fragment spectra (Fig 1).

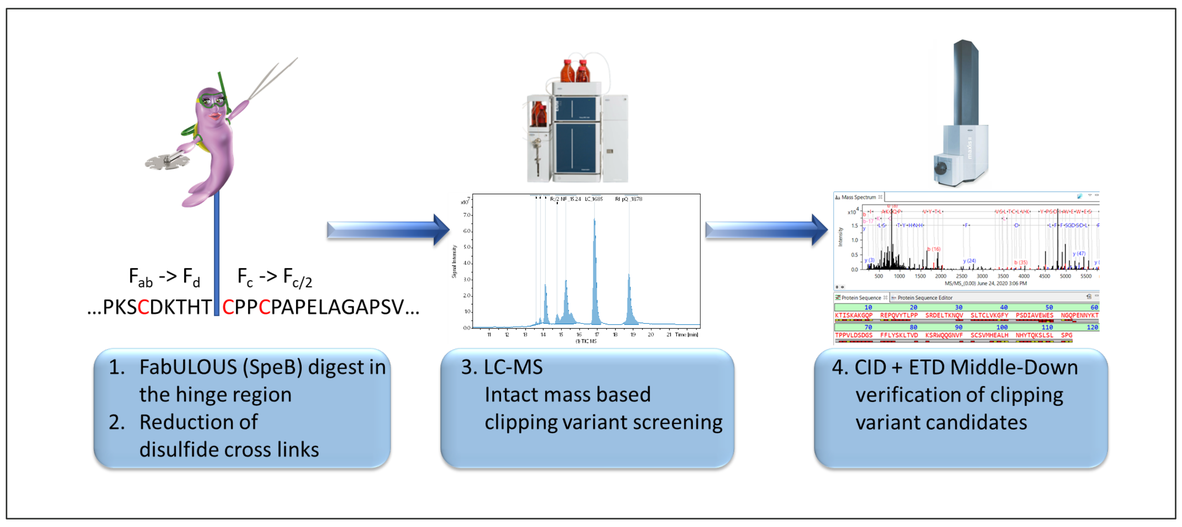

Fig. 1 Experimental design for the Vedolizumab protein clipping analysis. SpeB digestion produced Fab and Fc fragments. After reduction Fd, Fc, and LC subunits were analyzed by LC-MS, yielding the major expected subunits and some low abundant fragments. Protein clipping analysis in BioPharma Compass provided candidates based on accurate mass and isotope pattern. They were confirmed by direct sequence analysis of the clipping product candidates by ETD and CID

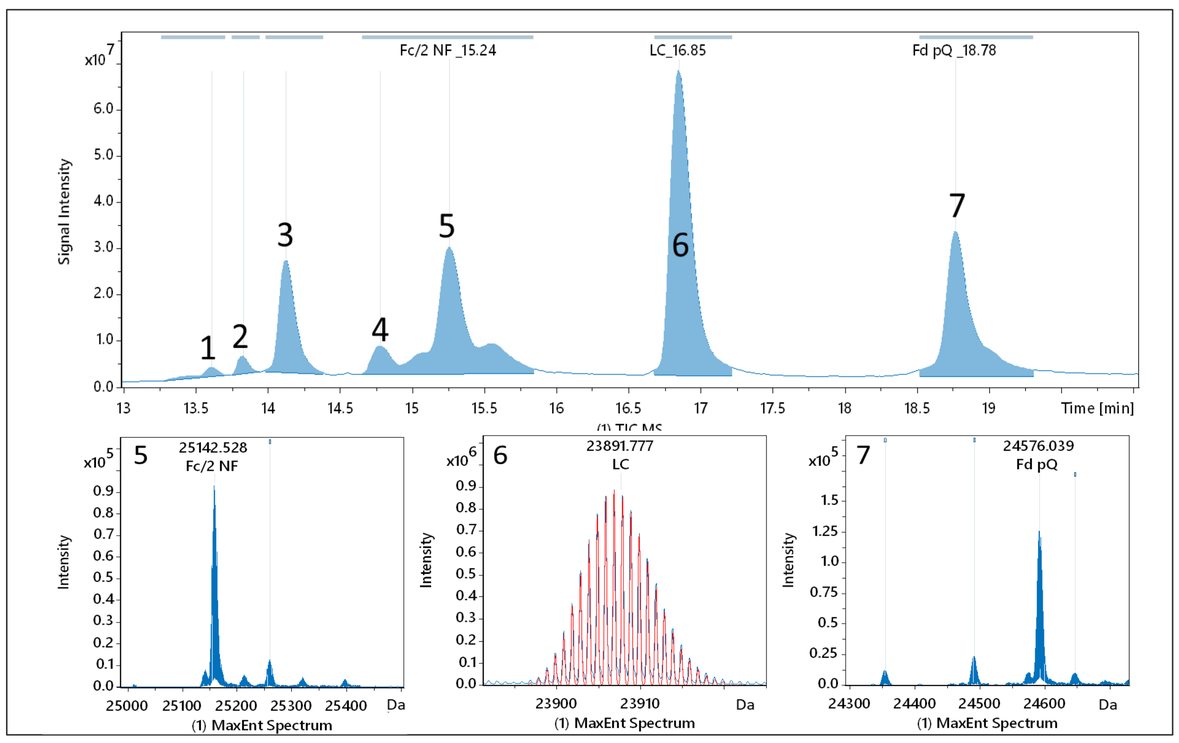

Fig. 2 Top: Total Ion Chromatogram of the SpeB-digested, reduced and deglycosylated Vedolizumab with the annotated subunits peaks 5-7. Peaks 1-4 were further characterized as putative protein clipping variants.
Bottom: MaxEnt deconvoluted MS spectra of chromatogram peaks 5-7, containing the IgGZERO deglycosylated Fc/2, the LC and pyro-glutamylated Fd subunit. All spectra were isotopically resolved and the calculated isotope patterns (red, LC in peak 6) show perfect alignment with the mass spectra. Monoisotopic molecular weights are annotated to the peaks.

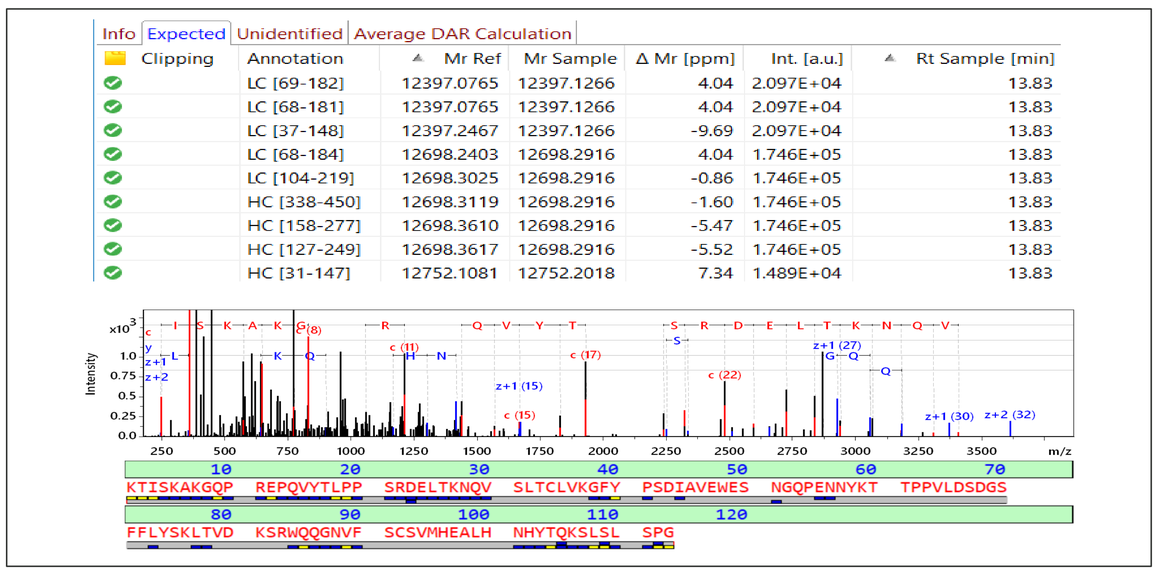

Fig. 3 Top: Proposed clipping variants in peak 2 after application of a 10-ppm mass tolerance. The 2 entries at 12698.3 Da. with < 2 ppm errors are the best candidates and were further analyzed by ETD and CID.
Bottom: ETD spectrum of the 12698.3 Da protein in peak 2 matched the sequence HC [338-450]. None of the other candidate sequences at 12698.3 Da were identified by Middle-Down sequencing

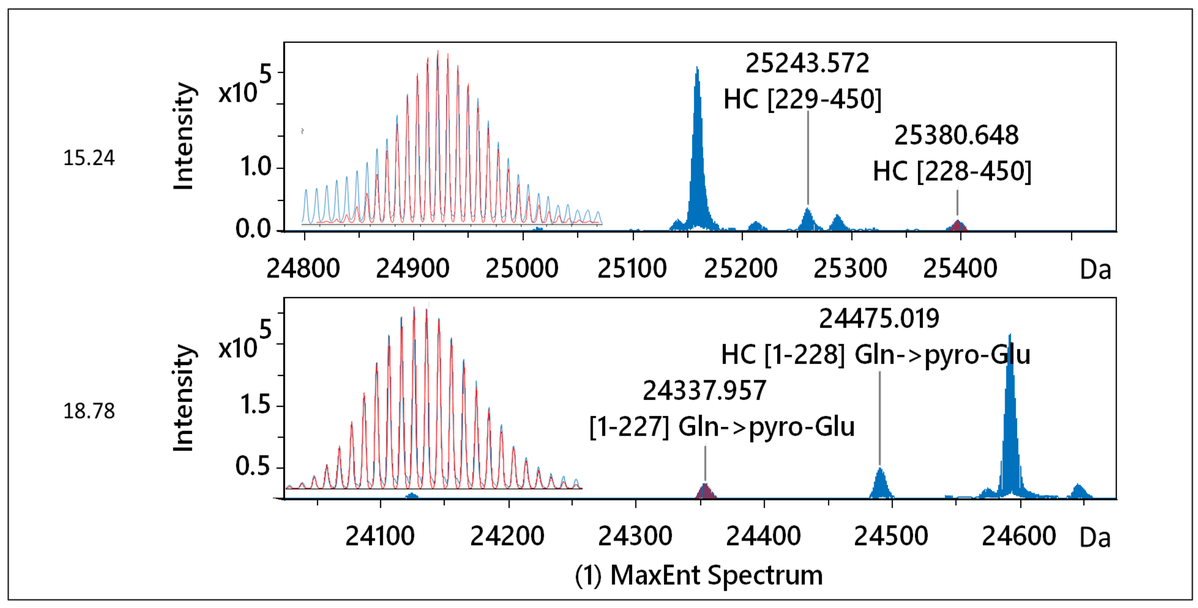

Fig.4. Intact mass spectra from chromatographic peaks 5 (top) and 7 (bottom). The annotated peaks show cleavage behind positions HC 227 and HC 228 and both complementary parts of the cleaved HC could be detected. All peaks show a perfect overlap with the calculated isotope pattern (red) with an average mass error of 0.8 ppm. The HC [228-450] was matched by the SNAP algorithm even against a significant background of chemical noise.

Results

- All expected subunits molecular weights (Fd, Fc/2 and Light Chain) were determined with a mass accuracy better than 2 ppm and the Middle-Down analysis provided an average sequence validation percentage (SVP) of 50 % from LC-ETD and 37% from LC-CID.
- Four additional chromatographic peaks were observed (*Fig. 2*). The Clipping Variant functionality of BioPharma Compass provided an initial list of vedolizumab clipping candidates based on the determined monoisotopic intact masses of these additional peaks.
- Different suggested clipping variants like HC [338-450], HC [342-450], and HC [238-450] could be confirmed by ETD- and CID-based Middle-down experiments whereas other suggested variants could be ruled out based on a missing fragment spectrum match (*Fig. 3*)
- the combined approach to qualify candidates by intact mass measurements for Middle-Down sequencing yielded more cleavage sites, 3 of which were confirmed by direct ETD and CID analysis, 2 were validated by their complementary nature and mass accuracy (Fig. 4)

Summary

- Three LC-MS runs of SpeB-digested and reduced Vedolizumab (LC-MS, LC-ETD, LC-CID) confirmed the expected cleavage site in the hinge region
- A workflow was described to identify antibody clipping variants based on subunit mass measurements and Middle-Down sequencing
- A mass accuracy of well below 2 ppm was important to provide sufficient specificity to define candidate sequences. This was achieved with monoisotopic mass assignments using the SNAP algorithm
- BioPharma Compass 2021 Clipping workflows, both for the intact mass-based prediction as well as for the Middle-Down sequencing facilitated the prediction and confirmation significantly to speed up potential drug substance clipping analysis

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