Streamlining mAb Characterization with a PASEF Based Disulfide Analysis Workflow

PROTEIN METRICS

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Overview

- Covalent disulfide bonds (DSBs) define the structure, function and stability of biotherapeutic proteins, such as monoclonal antibodies (mAbs).
- A common approach to elucidate them are tryptic digests under native and reduced conditions and to awkwardly compare those with significant manual validation.
- In this work we introduce a combination of improvements for **the near-automatic mapping of DSBs** based on these steps:
  - Generate **extensive MS/MS peptide maps** using PASEF on a trapped ion mobility QTOF
  - Apply bioinformatics for **the automated proposition of DSB-peptides** by MS and the confirmation by analysis of the MS/MS spectra.

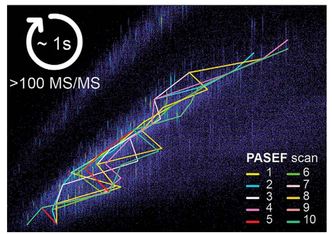

Fig. 1 PASEF Spectra were obtained with a standard 1.1 sec PASEF acquisition cycle. In this method, **P**arallel **A**ccumulation and **SE**rial **F**ragmentation results in increased sensitivity due to mobility focusing of the ions which are sequentially fragmented at > 100 Hz. This increased sensitivity and analysis depth results in more peptides, fragment ions and a high sequence coverage even for digests of non-reduced antibody samples.

Methods

Adalimumab was incubated for 3 h at 37 °C in the presence of 5.8 M Gua and 7 mM NEM, diluted 1 to 50 in digestion buffer and trypsin/LysC (Promega) was then added in a E/S 1:10 ratio followed by overnight digest at pH 5.8 in the presence of NEM.

Scrambling of Adalimumab DSBs was initiated by 10 or 30 min incubation at 70 °C before denaturation and digestion.

Peptides were separated on a 150 x 2.1 mm 1.7 µm C18 CSH column (Waters) using an Elute UHPLC coupled to a timsTOF Pro ion mobility QTOF mass spectrometer (both, Bruker Daltonics). A linear 60 minute gradient of 2-40% buffer B (100% ACN and 0.1% FA) was used, and PASEF scans (**Fig. 1**) were recorded and analysed.

Datasets were analysed using Byos software (Protein Metrics) built-in disulfide bond workflow.

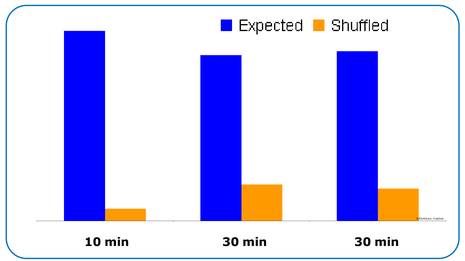

Fig. 2 Byos scrambling overview: The percentage of DSB-peptides in adalimumab obtained with expected and scrambled DSBs after 10 and 30 min.

Results

In the Byos disulfide bond workflow, MS/MS spectra are searched for DSB-peptides in an unbiased fashion. Inter- and intra-peptide DSBs between the heavy and light chain peptides are identified with <5% false discovery rate (**Fig. 3**). DSB-peptides were categorized as *expected* or *shuffled (scrambled)* linkages, and quantified by integrating their extracted ion chromatograms (EICs) (**Fig. 4**).

Byos MS/MS searches revealed ~98% sequence coverage (**Fig. 5**), including ~220 unique peptides and ~5000 PSMs per raw data file. In Adalimumab, disulfide bond scrambling was observed to be low, with >93% of all DSB-peptide EIC areas corresponding to expected linkages (**Fig. 2**).

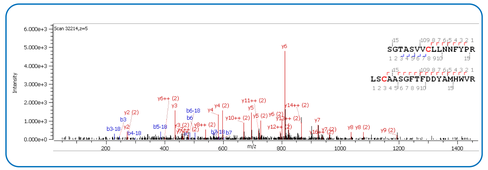

Fig. 3 PASEF spectrum of a scrambled Adalimumab DSB-peptide. The fragment ions are annotated and highlighted in the DSB-peptide sequence. Fragment ions with **(2)** annotations correspond to the second peptide chain (LSC…).

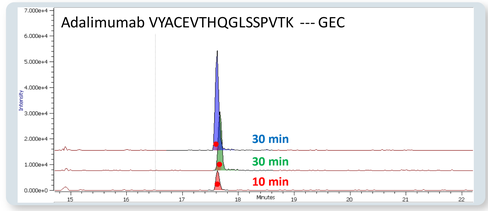

Fig. 4 EICs generated from a scrambled DSB-Peptide, indicating increased scrambling after 30 min vs. 10 min of heat treatment.

Disulfide scrambling was further investigated by artificial ageing experiments by heat treatment, which resulted in scrambled DSBs to increase to 16-18% of total DSB-peptides (**Fig. 2**) and scrambling hotspots were elucidated (**Fig. 6**).

Fig. 5 98 % sequence coverage of adalimumab LC and HC from PASEF analyses of a single proteolytic digest - incl. DSB-peptides after **10 min (red)** and **30 min (blue, green)** heat treatment.

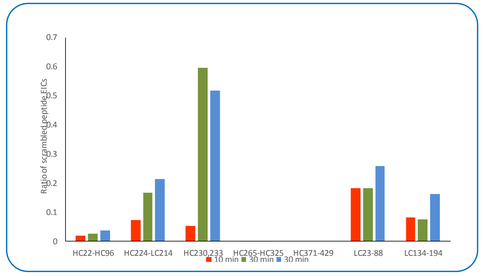

Fig. 6. Ratio of scrambled disulfide peptide to total observed disulfide peptides containing one of the pair of Cys residues. Induced scrambling is most apparent in inter-chain disulfide bonds HC-LC (224-214) and the HC hinge (230,233)

Conclusions

- The described approach yielded 98 % mAb sequence coverage from a single digest with PASEF analysis.
- Byos software confirmed the native DSBs in both mAbs and permitted the detection and identification of scrambled DSB-peptides down to < 0.01 % levels.
- Data analysis required 5 min operator time and ~2h computer time for searches, quantification and report generation.
- A single digest of the native protein was sufficient to provide a comprehensive analysis of disulfide structure of antibodies including the scrambled disulfide peptides.

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