A unified workflow for automatic mapping of disulfide bonds in protein therapeutics based on high resolution LC-QTOF

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Introduction

Disulfide bonds (DSB) are critical in maintaining a protein's three-dimensional structure, thus to preserve the protein functionality. Effective mapping of DSB in protein therapeutics provides insight into the drug integrity during the development process. Inappropriate DSB arrangement contributes to aggregation and must be monitored for patient safety.

The DSB mapping is analyzed by LC-MS via peptide mapping before and after reduction of the protein. In this study, we present a software workflow based on a high resolution QTOF platform for detection of disulfide bonds, disulfide scrambling and trisulfide bonds.

Methods

Rituximab was selected for testing. Before digestion, free cysteines were blocked by alkylation with N-Ethylmaleimide (NEM) in 50 mM phosphate buffer at pH 5.8. Denaturing agent Guanidine-HCl (8M) was added, the sample was buffer exchanged to 50 mM phosphate, and a Trypsin/ Lys C mixture was added for digestion. After digestion, the sample was divided into two fractions, one was reduced with DTT, and the other quenched with TFA.

The LC-MS analysis of the above two fractions were performed on a Bruker maXis II QTOF tandem mass spectrometer equipped with a Dionex Ultimate 3000 HPLC with gradient separation. The automated detection of disulfide bonds were performed by the PEAKS AB software from BSI.

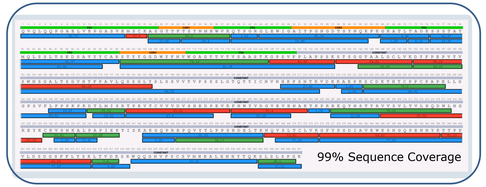

Fig. 1 Sequence coverage of Heavy Chain.

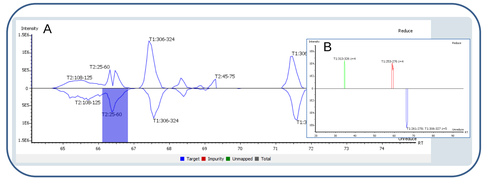

Fig. 2 Mirror plot of unreduced and reduced dataset.

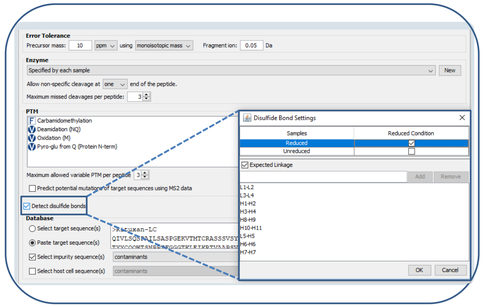

Fig. 3 Parameter settings for DSB automatic detection in PEAKS AB.

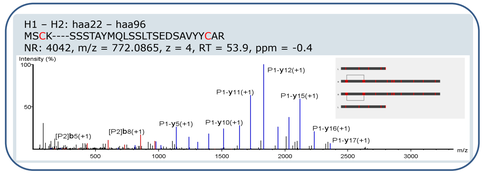

Fig. 4 Identified intra-chain DSB.

Table 1 Identified 9 DSBs of Rituximab.

No.	Peptide 1	Peptide 2	Retention Time (min)	Δ ppm	Scan No.
1	LC23: VTMTCR	LC87: VEAEDAATYYCQQWTSNPPTFGGGTK	51.59	-0.8	UR:3852
2	LC133: SGTASVVCLLNNFYPR	LC193: VYACEVTHQGLSSPVTK	62.82	-0.2	UR:4750
3	LC213: SFNRGEC	HC224: SCDK	3.71	-0.5	UR:336
4	HC22: MSCK	HC96: SSSTAYMQLSSLTSEDSAVYYCAR	53.9	-0.4	UR:4042
5	HC148: STSGGTAALGCLVK	HC204: SLSSVVTVPSSSLGTQTYICNVNHKPSNTK	54.15	-0.9	UR:4069
6	HC230, 233: SCDKTHTCPPCPAPE	HC230, 233: CPPCPAPELLGGPSVFLFPPKPK	75.16	0.8	UR:5674
7	HC265: TPEVTCVVVDVSHEDPEVK	HC325: CKVSNK	37.27	-1.3	UR:2791
8	HC371: NQVSLTCLVK	HC429: WQQGNVFSCSVMHEALHNHYTQK	53.39	-1.3	UR:3989

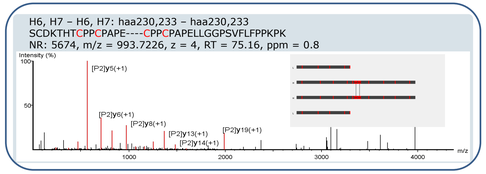

Fig. 5 Identified DSB in hinge region of Rituximab.

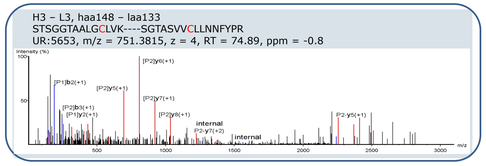

Fig. 6 Identified disulfide scrambling.

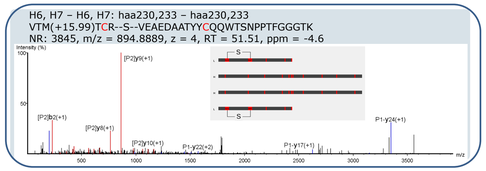

Fig. 7 Identified trisulfide bond.

Results

The amino acid sequence of Rituximab was validated with the unreduced peptide map and the sequence coverage of the heavy and light chains were more than 99 % (**Fig. 1**). The chromatograms of reduced and unreduced fractions were overlaid in mirror plots with the tools in PEAKS AB. The differences can be found in the chromatograms that result from the reduced disulfide bonds (**Fig. 2A**). The DSB peptide can be observed as one peak in the chromatogram of unreduced sample and two peaks (two separate peptides) in the reduced sample (**Fig. 2B**).

The parameter settings for the DSB automatic detection in PEAKS AB are shown in **Fig. 3**. After the DSB search was complete, all 9 expected and nonredundant DSBs of Rituximab were identified automatically by MS/MS data, including intra-chain DSBs (**Fig. 4**), inter-chain DSBs (data not shown) and hinge region DSBs (two DSBs in hinge region were identified in one DSB peptide (**Fig. 5**). The data from the unreduced sample also yielded very small amounts of disulfide scrambling (**Fig. 6**) and trisulfide bonds (**Fig. 7**). After the database search, a table containing all expected DSBs was generated (**Table 1**), the mass error shows a good mass accuracy of maXis II QTOF.

Conclusions

- All 9 expected and nonredundant DSBs of Rituximab were identified automatically by the Peaks AB software workflow based on the high resolution maXis II Q-TOF. Meanwhile, more than 99 % amino acid sequence coverage was obtained.
- Two other unexpected sulfur bridges, disulfide scrambling and trisulfide bond, can also be identified automatically by this workflow.
- The combination of good mass accuracy data from maXis II QTOF and automated detection software of PEAKS AB provides high confidence and time-saving in disulfide bonds detection.

maXis II Q-TOF