Confirmation of disulfide linkages in adalimumab using electron activated dissociation (EAD)

Featuring the SCIEX ZenoTOF 7600 system with EAD and Protein Metrics Inc. software

Zoe Zhang[1], Jason Causon[2], Pavel Ryumin[2], Takashi Baba[2], Bill Loyd[2], Kerstin Pohl[1]
[1]SCIEX, US; [2]SCIEX, Canada

The data presented in this technical note show the confirmation of disulfide-linked peptides alongside the confirmation of the sequence for a biotherapeutic monoclonal antibody (mAb). The analysis of disulfide-linked peptides was performed using rapid data dependent acquisition (DDA) and automated data interpretation with Protein Metrics Inc. software as part of a disulfide mapping analysis. With this workflow, regular and advanced characterization leveraging the novel electron activated dissociation (EAD)-based fragmentation[1, 2] is achievable in one injection, enabling a streamlined characterization accessible to every user level.

Ensuring drug safety and efficacy is essential for biotherapeutics and drives the need for in-depth characterization during their development. This includes the identification and the localization of post-translational modifications (PTMs), especially the characterization of disulfide linkages. Since disulfide bonds are critical for maintaining the three-dimensional structure and function of biomolecules[3], elucidating the connections between cysteine residues is essential for validating disulfide design and understanding the higher order structure of biotherapeutics. Different analytical and biophysical methods have been employed for the analysis of disulfide linkages, such as Edman degradation.[4] With the wide adoption of mass spectrometry,

bottom-up approaches with tandem mass spectrometry have become the method of choice.[5] However, disulfide bonds prevent effective fragmentation around the linked cysteine residues in collision induced dissociation (CID), which limits the information available for identifying the disulfide-bound peptides as well as disulfide patterns. Although alternative fragmentation mechanisms exist, these techniques can suffer from long reaction times, low sensitivity and lack of reproducibility.

Here, a new fragmentation type based on EAD[1, 2] is demonstrated for the confirmation of disulfide-linked peptides from a commercial mAb. The data were acquired using an untargeted 10 Hz DDA method and interpreted with Protein Metrics Inc. software. This workflow demonstrates a routine approach for the streamlined characterization of disulfide-linked peptides with a new level of data quality.

Key features of EAD with the SCIEX ZenoTOF 7600 system

- **New depths of peptide mapping analysis:** EAD with fast DDA enables alternative fragmentation for routine, in-depth analysis of next generation protein therapeutics and standard mAbs

- **Higher levels of structural information:** Changing the mechanism of fragmentation by tuning the electron energy may provide a higher level of structural information, particularly for disulfide linked peptides and sequence confirmation

- **Higher MS/MS sensitivity:** Increased detection of fragments (5 to 10 fold) using the Zeno trap enables higher confidence in data assignment

- **High reproducibility:** Reproducible fragmentation with EAD for singly, doubly, and multiply charged ions enables analysis of more precursors than other alternative and low reproducibility fragmentation techniques

- **Streamlined and easy-to-use:** Fully automated data acquisition in DDA mode using EAD with SCIEX OS software, and automated data interpretation with Byos software (Protein Metrics Inc.) simplifies the entire user experience

Figure 1. The SCIEX ZenoTOF 7600 system.

Methods

Sample preparation: The mAb sample (adalimumab) was denatured with 7.2M guanidine hydrochloride in 50mM Tris buffer (pH 7.0), free cysteine was capped with 5mM iodoacetamide. Digestion was performed with trypsin/Lys-C enzyme at 30 °C for 16 h. The reaction was stopped with 1% formic acid.

Chromatography: 3 µL (4 µg) of the trypsin/Lys-C digest were separated with a CSH C18 column (2.1×100 mm, 1.7 µm, 130 Å, Waters) using an ExionLC AD system. The mobile phase A consisted of water with 0.1% formic acid, while the organic phase B was acetonitrile 0.1% formic acid. A gradient profile was used at a flow rate of 350 µL/min (Table 1). The column temperature was maintained at 50°C.

Table 1. Chromatography for peptide mapping analysis.

Time [min]	Mobile phase A [%]	Mobile phase B [%]
Initial	98	2.0
1.00	98	2.0
86.00	55	45
86.50	20	80
87.00	2.0	98
87.50	2.0	98
87.51	98	2.0
90.00	98	2.0

Mass spectrometry: Data were acquired with an information dependent acquisition (IDA) method using the SCIEX ZenoTOF 7600 system. The electron energy for the alternative fragmentation in the EAD cell was set to a value of 7 eV. Detailed method parameters are summarized in Table 2.

Table 2. MS parameters.

Parameter	MS	MS/MS
Scan mode	TOF-MS	IDA dependent
Polarity	positive	
Gas 1	50 psi	
Gas 2	50 psi	
Curtain gas	35 psi	
Source temperature	550 °C	
Ion spray voltage	5500 V	
Declustering potential	80 V	
Collision energy	12 V	
CAD gas	7	
Maximum candidate ion	10	
Intensity threshold	125 cps	
Charge states	2 to 10	
Exclusion time	6 s after 2 occurrences	
Start mass	200 m/z	100 m/z
Stop mass	2,000 m/z	3,000 m/z
Electron KE	NA	7 eV
Electron beam current	NA	4750 nA
ETC	NA	100
Zeno trap	NA	ON
Accumulation time	0.25 s	0.10 s
Time bins to sum	8	12

Data processing: Data were processed using Byos software (Protein Metrics Inc.).

The what, why and how

Disulfide bonds are a common post-translational modification found in globular proteins that have been comprehensively studied since the 1980s.[6] Because they are tightly related to protein folding structure, they can affect both the efficacy and safety of a biotherapeutic. With the advancement of modern mass spectrometry, bottom-up approaches have become the method of choice for the characterization of product quality attributes, enabling the simultaneous identification and localization of modifications including disulfide bonds. Peptide mapping analyses with collision induced dissociation (CID) are commonly utilized to investigate disulfide formation. However, the full characterization of disulfide-linked peptides is still a challenge. With CID the peptide backbone generally fragments (Figure 2), but the disulfide bonds rarely break, which can lead to a complicated yet incomplete spectrum that is difficult to interpret. Thus, a combination of multiple enzymes and even partial reduction has been explored in sample preparation to clarify the ambiguity in the characterization. These complex strategies are both time and sample consuming, and therefore expensive while successful characterization has a mixed probability.

In contrast to CID fragmentation, multiple publications show that alternative fragmentation, such as electron capture dissociation (ECD), preferentially breaks disulfide bonds over peptide backbones.[7,8] Similarly, EAD also shows a preference for breaking disulfide bonds with the great benefit of it being applicable for a general peptide mapping analysis.[9, 10] The process of disulfide linkage dissociation is shown in Figure 3. EAD derived MS/MS data provide greater confidence in the identification of the peptides linked by disulfide bonds as those peptides can be more fully sequenced after the cleavage of the
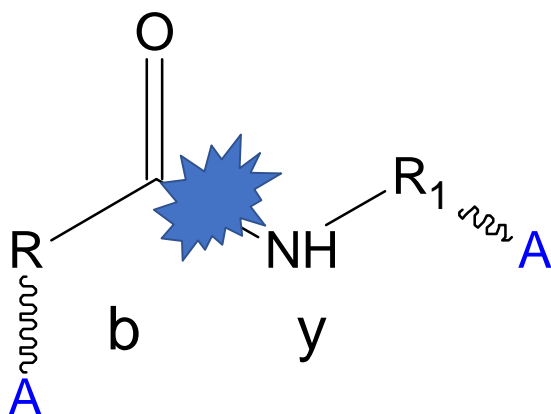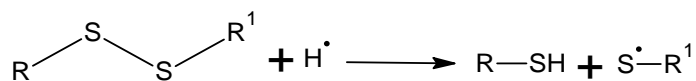

Figure 3. Dissociation of disulfide bonds by EAD.

disulfide bond.[7] In addition, the mass information of each individual peptide provides further confirmation.

Although alternative fragmentation techniques have been used for this type of identification in the past, their adoption by the biopharmaceutical industry has been limited by their overall low sensitivity and lack of automated DDA workflows.

With the SCIEX ZenoTOF 7600 system, an alternative fragmentation mechanism is introduced, enabling scientists to get an in-depth picture of their samples by using analytical flow liquid chromatography (LC) separation in combination with a fast scanning DDA method and automated processing using Protein Metrics Inc. software. This breakthrough technology realizes the dream of high-throughput fragmentation in the biopharmaceutical industry, capable of answering complex questions in a routine manner.

Characterization of disulfide-linked peptides

This study focused on the characterization of a commercialized mAb, adalimumab, an IgG1 antibody. Adalimumab contains 16 disulfide bonds which are critical for maintaining its three dimensional structure. Using intact mass analysis, the existence of these disulfide bonds was confirmed.[11] However, this approach cannot provide detailed information on whether cysteine residues are connected as designed.

Here, a DDA approach in combination with Zeno EAD is used for routine non-reduced peptide mapping analyses. EAD fragmentation enables advanced characterization during DDA acquisition while the Zeno trap enhances the detection of fragment ions and thus the correct identification of large disulfide peptides. This approach allows for the straightforward data interpretation using Byos software (Protein Metrics Inc.).

Figure 4 shows an example of an EAD spectrum for a disulfide linked peptide. The four most dominant fragments observed in the spectrum are the +1 and +2 charge states of the two linked peptides (SGTASVVCLLNNFYPR and VYACEVTHQGLSSPVTK) after the cleavage of the disulfide bond with EAD. The high resolution accurate mass data therefore provides clear information on which two peptides are connected by a disulfide bond. In addition, a great MS/MS amino acid coverage of the peptides was achieved with EAD, further enhancing the confidence in the assignment (86.7% for
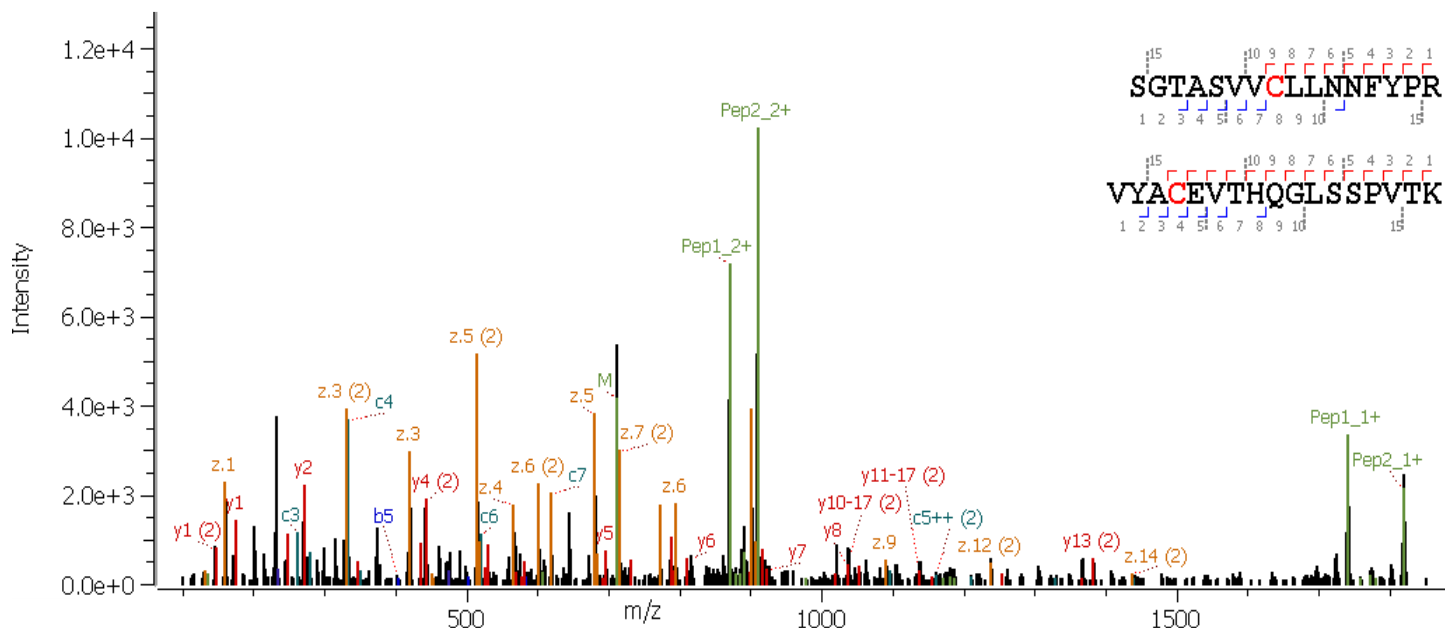

Figure 2. Schematic for CID peptide fragmentation. Mainly *b* and *y* fragment ions are produced from the peptide backbone during CID.

Figure 4. MS/MS spectrum for a disulfide-linked peptide SGTASVVCLLNNFYPR/VYACEVTHQGLSSPVTK using EAD.

SGTASVVCLLNNFYPR and 93.7% for VYACEVTHQGLSSPVTK). Traditionally, disulfide-bonded peptides suffered quite often from low MS/MS scores for either both linked peptides or one of the peptides using CID due to the poor fragmentation. In contrast, EAD provides an incredibly high quality spectra for both linked peptides. For the peptide in Figure 4, high MS/MS scores of 590 and 418 for were obtained for SGTASVVCLLNNFYPR and for VYACEVTHQGLSSPVTK, respectively.

Figure 5 shows a comparison between CID (A) and EAD (B) for a disulfide linked peptide. Although the linked peptides are significantly different in size, the amino acid coverage achieved with CID was 81%. Using a standard energy of 7 eV for EAD during a general peptide mapping analysis, the coverage could be further enhanced to an excellent 100% for the same linked peptides. With CID, the disulfide linkage doesn't break and the existence of the intact disulfide bond prevents fragmentation along the peptide backbone close to the linkage. Peptides with multiple disulfide bonds show an even more profound effect with even less fragment coverage with CID. In contrast, EAD favors cleavage of disulfide bonds and provides higher coverage compared to CID. The dissociation of the disulfide bond results in the detection of individual peptides (NQVSLTCLVK as z=+1 and WQQGNVFSCSVMHEALHNHYTQK as z=+2 and z=+3, for example), which enhances the confirmation of which peptides are being connected. EAD further produces rich fragmentation for both peptides, giving 100% amino acid coverage for the peptide NQVSLTCLVK and for the peptide WQQGNVFSCSVMHEALHNHYTQK. In addition to the excellent

sequence coverage, the spectrum also shows two diagnostic ions from two Leu residues (z6-43++, z3-43), which can be directly used to discriminate Leu from Ile as discussed in a previous technical note.[12]

Unambiguous characterization of disulfide peptides in one single DDA run with automated data interpretation was successfully achieved with the SCIEX ZenoTOF 7600 system. This is an example of how to achieve high confidence analysis not only in sequence confirmation but also in disulfide linkage confirmation. Even though disulfide linkage analysis by LC-MS/MS has been a challenge for years, the workflow demonstrated here can be accomplished in a reproducible manner using Zeno EAD. This strategy can also be applied for routine peptide map analysis, enabling biotherapeutics sequence confirmation, disulfide characterization, PTM analysis and isomer differentiation such as leucine and isoleucine in a single injection.
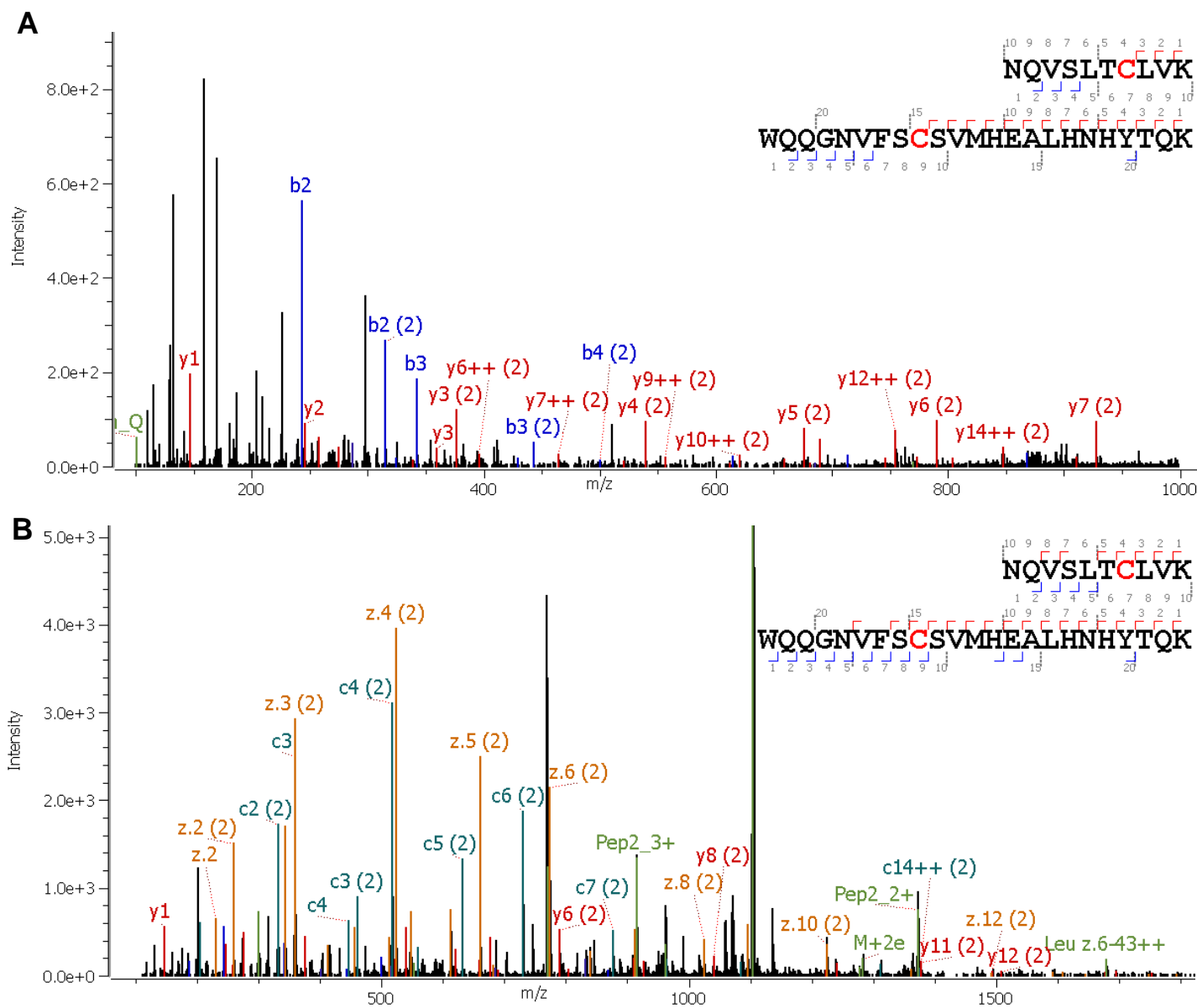
Figure 5. MS/MS spectra for a disulfide-linked peptide NQVSLTCLVK/WQQGNVFSCSVMHEALHNHYTQK. (A) CID spectrum (B) EAD spectrum.

Conclusions

- Confident sequence and disulfide linkage confirmation was achieved with EAD, a novel fragmentation technique
- MS/MS fragment detection was significantly enhanced when using the Zeno trap compared to traditional high-resolution MS/MS analysis. This enabled confident fragment assignment with excellent data quality, even for precursors with medium or very low intensities, such as modified peptides.

- The robust, reproducible and easy-to-use alternative fragmentation enables users to directly answer challenging analytical questions with the SCIEX ZenoTOF 7600 system and SCIEX OS software
- Automatic data processing enables the routine and advanced characterization of complex biotherapeutics and standard mAbs in a reproducible manner, using Protein Metrics Inc. software

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