Comprehensive mapping of disulfide linkages in etanercept using an electron activated dissociation (EAD) based LC-MS/MS methodology

Zhengwei Chen and Lei Xiong
SCIEX, Redwood city, USA

ABSTRACT

The disulfide linkages of etanercept, a dimeric fusion protein, were characterized utilizing both collision induced dissociation (CID) and electron activated dissociation (EAD) in combination with a multi-enzyme approach (trypsin + Glu-C). The performance of CID and EAD in terms of peptide backbone coverage for disulfides at different complexity level were compared. For simple disulfides with relatively short peptides, both CID and EAD works equally well in generating fragments for good peptide backbone sequence coverage. However, when the length of peptides increases, EAD provides unique benefits in generating wealth of fragments for every peptide involved in the disulfides. Particularly, for disulfide peptides containing more than 2 disulfide bonds, CID generated very limited peptide backbone fragments; whereas EAD generated abundant fragments for the solid identification of every peptide. In addition, as a soft fragmentation technique, EAD also was able to successfully preserve the labile O-glycan on the disulfides, providing additional benefit of accurately locating the O-glycosylation site.

INTRODUCTION

Disulfide linkages in biotherapeutic proteins impact their conformation and therefore are considered critical quality attributes (CQAs). LC-MS based analytical characterization is of vital importance in confirming the disulfide linkages as well as the sequence of the associated peptides. Etanercept contains 26 intra-chain and 3 inter-chain disulfide bonds. As a result, up to 4 peptides can form an extremely complex and large disulfide peptide with multiple disulfide linkages, even when using multi-enzyme digestion. Widely used CID provides very limited sequence information for such peptides. To overcome this challenge, a type of hot electron-capture dissociation (ECD), namely EAD,[1,2] was investigated with regards to dissociation of disulfide bonds, which allows every peptide involved in the disulfide linkage to be confidently sequenced.[3,4] Data processing and confident annotation of alternative fragmentation data can pose a bottle neck, especially in the case of complex disulfide-bonded peptides. To this end, the Biologics Explorer software filled the gap by providing excellent fragment annotations including *a/x*, *b/y*, *c/z* ions generated from EAD. For the disulfides that contained more than 2 peptides, the MS/MS spectrum of every peptide was well annotated, allowing solid sequencing of the peptide.

MATERIALS AND METHODS

Sample preparation: Etanercept, a dimeric fusion protein of approximately 125 kDa, which consists of 2 extracellular domains of the tumor necrosis factor receptor 2 (TNFR2) and the Fc part of human IgG1, was denatured with 7 M guanidine-hydrocholoride (GuaHCl) in 100 mM Tris, pH 7.0 and alkylated with 20 mM iodoacetamide. Then the sample solution underwent buffer exchange to 1 M GuaHCl using a 10 kDa molecular cut-off filter followed by trypsin/Lys-C combined with Glu-C digestion overnight at 37ºC. The digestion was quenched with trifluoroacetic acid.

Chromatography: LC analysis was performed with an ExionLC system (SCIEX). The protein digest was injected onto an ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å, Waters) with column temperature set to 45ºC. The LC method was 60 min with 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile as mobiles phases at flow rate of 0.2 mL/min.

Mass spectrometry: LC-MS/MS data were acquired with data dependent acquisition in positive ion mode on the ZenoTOF 7600 system (SCIEX) using SCIEX OS software 2.1. CID and EAD MS/MS data were acquired in separate injections to allow a comparison. Source and TOF MS parameters were kept the same for CID and EAD. Key TOF MS and MS/MS parameters are listed in table 1-2.

Data analysis: Data were processed using the peptide mapping workflow embedded in SCIEX Biologics Explorer software. The annotation of EAD data was well supported by default settings optimized for SCIEX EAD data in the software. The expected disulfides linkages were set as fixed modification. For the analysis of peptides modified with O-glycans, the embedded O-glycan library was used. All the figures presented here were directly exported from Biologics explorer software.

Table 1. TOF MS parameters.

Parameter	Value	Parameter	Value
Ion source gas 1	*50 psi*	*Source temperature*	*450°C*
Ion source gas 2	*50 psi*	*Ion Spray voltage*	*5500 V*
Curtain gas	*35 psi*	*CAD gas*	*7*
MS range	*300–2000 m/z*	*Accumulation time*	*0.2 s*
Delustering potential	*80 V*	*Time bins to sum*	*8*

Table 2. TOF MS/MS parameters for CID and EAD.

Parameter	Value	Parameter	Value
MS range	*100–2000 m/z*	*Time bins to sum*	*10*
Delustering potential	*80 V*	*Exclusion width*	*6 s for 2 occurrences*
#MS/MS per cycle	*15*	*Zeno trap*	*On*
Q1 resolution	*Unit*	*Zeno trap threshold*	*100,000*
CE for CID	*Dynamic collision energy*	*EAD parameters*	*KE: 7 eV Beam current: 5500 mA Reaction time: 10 ms*

RESULTS

The most complex disulfide linkage of etanercept lies in the TNFR region with a couple of peptides connected by double or even triple disulfide bonds. CID only provided very limited peptide backbone information, hindering its ability for the unambiguous confirmation of each peptide. In contrary, EAD generated rich *c/z* and *b/y* fragments for each peptide, providing confident sequence confirmation. The disulfide linkages in the Fc domain are less complex. However, up to 11 O-glycosylations on serine/threonine sites exist in this region, impeding the analysis. Here, EAD enabled the analysis of site-specific O-glycosylated disulfide peptides by preserving the labile O-glycan, providing highly confident results of both O-glycans and disulfide linkages.

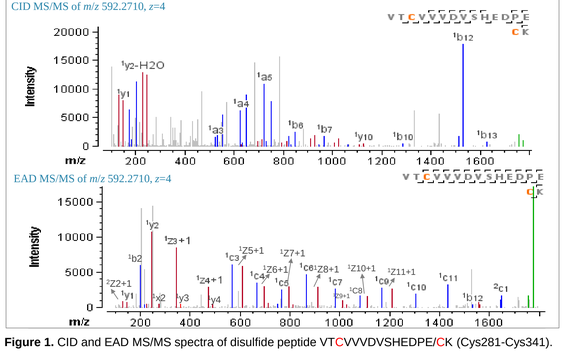

Figure 1. CID and EAD MS/MS spectra of disulfide peptide VTCVVVDVSHEDPE/CK (Cys281-Cys341).

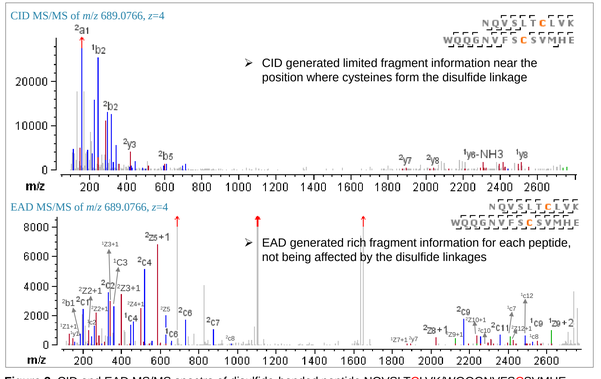

Figure 2. CID and EAD MS/MS spectra of disulfide-bonded peptide NQVSLTCLVK/WQQGNVFSCSVMHE (Cys387-Cys445).

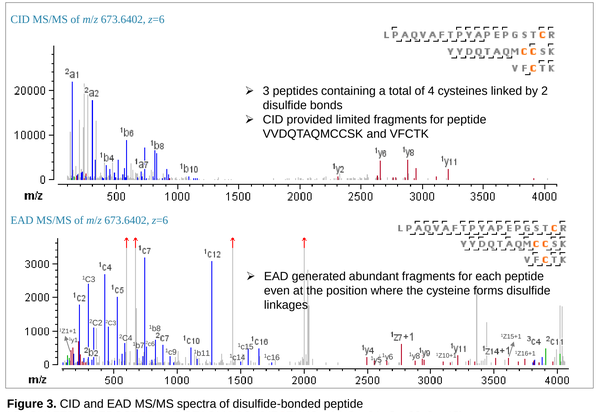

Figure 3. CID and EAD MS/MS spectra of disulfide-bonded peptide LPAQVAFTPYAPEPGSTCR/YYDQTAQMCCSK/VFCTK (Cys18-Cys31, Cys32-Cys45).

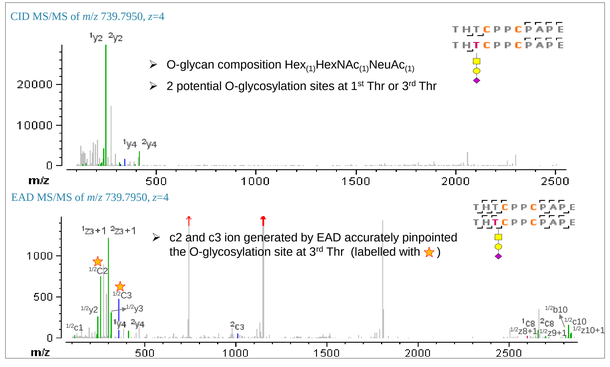

Figure 4. CID and EAD MS/MS spectra of disulfide-bonded peptides THT*CPPCPAPE/ THT*CPPCPAPE(Cys246-Cys246, Cys249-249), ★ O-glycosylation site.

CONCLUSIONS

- EAD provided solid sequence and disulfide linkages confirmation, especially for long disulfide peptides
- Peptides that contain 2 or more disulfide bonds, EAD generated a wealth of fragments for reliable sequencing of every peptide involved in the disulfides, showing superior fragmentation coverage compared to CID
- Additionally, EAD preserves the labile O-glycan, enabling site-specific characterization of O-glycosylated disulfide-bonded peptides
- EAD provides solid backbone sequencing for disulfide peptides while enabling unambiguous localization of the O-glycosylation site, simultaneously

REFERENCES

1. Baba T et al. (2015) Electron capture dissociation in a branched radio-frequency ion trap, *Anal. Chem.*, **87**(1): 785−792.
2. Baba T et al. (2021) Dissociation of biomolecules by an intense low-energy electron beam in a high sensitivity time-of-flight mass spectrometer. *J. Am. Soc. Mass Spectrom.* **32**(8):1964-1975.
3. Confirmation of disulfide linkages in adalimumab using electron activated dissociation, SCIEX Technical note, RUO-MKT-02-12913-B.
4. Comprehensive characterization of complex linkage structures in a bispecific monoclonal antibody (mAb) using electron activated dissociation (EAD), SCIEX Technical note, RUO-MKT-02-14373-A.

TRADEMARKS/LICENSING

To receive a copy of this poster:

- Scan the code with your phone camera
- Complete the form