A complete solution for sequence variant analysis of recombinant antibodies and therapeutic proteins with electron-activated dissociation (EAD) and automatic data processing

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ABSTRACT

Sequence variants (SVs) have increasingly gained attention from regulatory agencies and the biopharmaceutical industry, given their potential impact the efficacy and safety of biotherapeutics. LC-MS/MS is a powerful technique to identify SVs and provide the accurate localization of these substitutions. However, the commonly used collision-induced dissociation (CID) method fails to differentiate amino acids that have isomer pairs, such as leucine (Leu) and isoleucine (Ile) or aspartic acid (Asp) and iso-aspartic acid (isoAsp). Instead, electron-activated dissociation (EAD) is a powerful tool to differentiate these isomers.[1,2] In addition, the many possible variants per substitution complicate data processing for SVs. An automatic data processing approach that can overcome this challenge is integrated into the workflow presented here.[3,4]

INTRODUCTION

The occurrence of sequence variants (SVs) resulting from inadvertent amino acid substitutions during the production of therapeutic proteins has recently become a focal point of interest for regulatory agencies and the biopharmaceutical industry, as they can affect both efficacy and safety. Typically, with well-optimized production systems, SVs are present at minimal levels in the final protein products due to the high accuracy of DNA replication and protein biosynthesis processes in mammalian expression systems such as Chinese hamster ovary cell lines. However, if the production cell line chosen has unexpected DNA mutations or if the manufacturing process is not fully optimized, resulting in a depletion of certain amino acids in the cell culture media in bioreactors, the levels of these SVs can be significantly elevated. Thus, it is imperative to design and implement an effective monitoring and control strategy to prevent or minimize the potential risks of SVs during the early stages of product and process development. Here, we leveraged EAD to generate diagnostic ions for Leu/Ile differentiation by generating signature ions. Notably, the level of this SV is 0.02% compared to its native form, suggesting that EAD is a sensitive enough tool to provide confident identification for extremely low abundant SVs.

MATERIALS AND METHODS

Sample preparation:
Etanercept is an approximately 125 kDa dimeric fusion protein that consists of 2 extracellular domains of the tumor necrosis factor receptor 2 (TNFR2) and the Fc region of human IgG1. The protein standard was denatured with 7M guanidine-HCl in 100mM Tris at pH 7.0, reduced with 10mM dithiothreitol (DTT) and then alkylated with 20mM iodoacetamide (IAM). The sample solution was buffer exchanged to 1M guanidine-HCl by a 10K molecular cut-off filter. The solution was then digested overnight at 37°C with trypsin/Lys-C combined with Glu-C. The digestion was quenched with trifluoroacetic acid (TFA). The digested sample was subjected to LC-MS/MS analysis using an analytical flow HPLC coupled with an accurate mass QTOF instrument.

HPLC conditions:
LC analysis was performed with an ExionLC system. Protein digest was injected onto an ACQUITY CSH C18 column (2.1 x 150 mm, 1.7 µm, 130 Å, Waters) with column temperature set at 45°C. The LC gradient was 60 min, mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile with a flow rate of 0.2 mL/min.

MS/MS conditions:
LC-MS/MS data-dependent acquisition (DDA) data were acquired in positive mode with a SCIEX ZenoTOF 7600 system using SCIEX OS 2.1. CID and EAD MS/MS data were acquired for comparison. Source and MS1 parameters were kept the same for CID and EAD. Key TOF MS/MS parameters are listed in Tables 1 and 2.

Data analysis:
Data were processed using the peptide mapping workflow embedded in SCIEX Biologics Explorer with the sequence variants function enabled. The EAD fragmentation annotation was well-supported by Biologics Explorer software and the EAD preset was selected for EAD data processing. All the figures presented here were directly exported from Biologics Explorer software.

Table 1. TOF MS parameters.

Parameters	Value	Parameters	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 psi*
MS range	*300-2000 m/z*	*Accumulation time*	*0.2 s*
Delcustering potential	*80 V*	*Time bins to sum*	*8*

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

Parameters	Value	Parameters	Value
MS range	*100-2000 m/z*	*Time bins to sum*	*10*
Delustering Tpotential	*80 V*	*Exclusion width*	*6 s for 2 occurrences*
#MS/MS per cycle	*15*	*Zeno Pulsing*	*On*
Q1 resolution	*Unit*	*Zeno Threshold*	*100000*
CID parameters	*Dynamic collision energy*	*EAD parameters*	*KE: 7 eV Beam current: 5500 mA Reaction time: 10 ms*

RESULTS

There are 467 amino acids on each chain of etanercept and any given amino acid could be substituted with another one in the bioreactor manufacturing system during the protein biosynthesis process. A reduced peptide mapping was performed to provide sequence information at the amino acid level to identify possible SVs. Both CID and EAD approaches provided close to 100% sequence coverage for the native peptides through a regular peptide mapping search. To provide a complete understanding of the SV landscape of etanercept, 378 possible SVs were incorporated into the software and searched against the dataset to create an SV-centric targeted workflow.

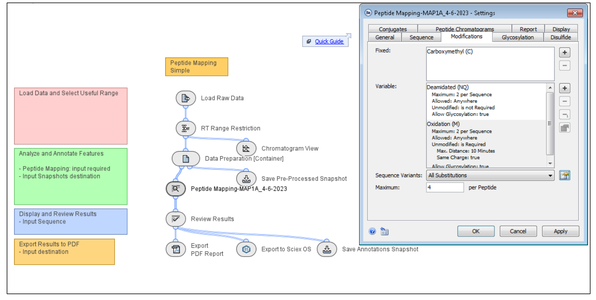

Figure 1. SV-centric targeted workflow for peptide mapping in Biologics Explorer software.

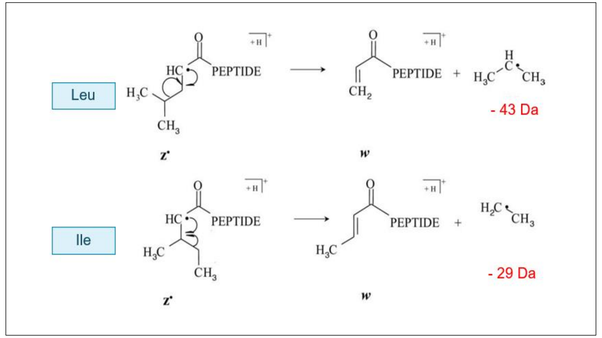

Figure 2. Fragmentation mechanism for the formation of diagnostic ions for Leu and Ile. Leu produced a diagnostic ion at m/z z-43 and Ile produced a diagnostic ion at m/z z-29 under EAD.

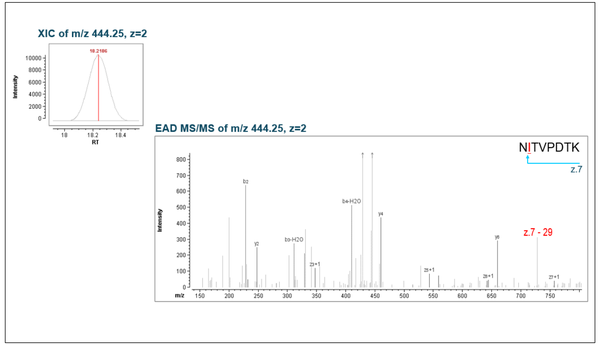

Figure 3. The presence of z7-29 ion and z7-43 ion produced helps to confirm the second amino acid from the N-terminal is an Ile, not Leu.

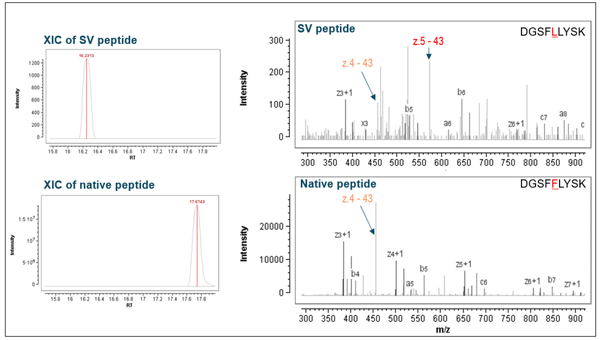

Figure 4. Trace levels of SV at 0.02% compared to the native peptide were detected for etanercept in the Fc region for peptide DGSFFLYSK. The SV was identified as a substitution with Leu or Ile on the F located at the sixth position from the N-terminal by both CID and EAD through the y5/y6 and z5/z6 ions.

CONCLUSIONS

- EAD technology offers powerful differentiation between amino acid isomers to analyze SVs, such as F to Ile or Leu substitutions
- Biologics Explorer software offers an automatic highly robust SV-centric peptide mapping searching workflow for high-throughput screening of the sequence variants mAb
- As low as 0.02% sequence variant (F->Leu) was detected, demonstrating the superior sensitivity of the SCIEX ZenoTOF 7600 system

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