Post-translational modification (PTM) profiling on fusion protein aflibercept using a novel fragmentation technique

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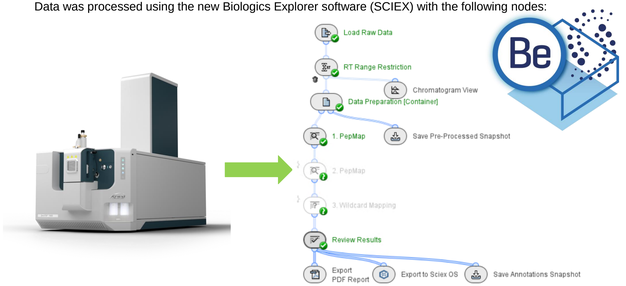
INTRODUCTION

Aflibercept is an Fc fusion protein used to treat wet macular degeneration and metastatic colorectal cancers. It contains five different N-linked glycosylation sites with extensive glycan distribution, which exhibit additional levels of complexity in terms of characterization. Glycosylation including glycan structures and glycan occupancy, plays a vital role in stability and efficacy of a biotherapeutic. Therefore, the glycan profile is often considered a critical quality attribute (CQA). Bottom-up workflows are frequently used for glycopeptide characterization as protein sequence information and glycan profiling can be obtained, simultaneously. However, traditionally used collision-induced dissociation (CID) struggles to provide accurate information on glycosylated peptides. Alternative fragmentation on the other hand is challenged by providing such information easily and reproducibly in order to be used routinely within the biopharma industry. Here within, the ability of electron activated dissociation (EAD) for peptide mapping MS/MS analyses is examined.

EAD, a newly developed fragmentation technique unique to the ZenoTOF 7600 system (SCIEX), allows for optimized peptide fragmentation with a platform method.[1,2] This allows for accurate localization of the linked glycans alongside confident identification of the peptide through high MS/MS sequence coverage of the peptide backbone.[3,4] In addition, Zeno EAD enables fast and highly sensitive data-dependent acquisition (DDA). This approach further overcomes long existing challenges of alternative fragmentation, such as long reaction times, limited charge state fragmentation, and low sensitivity. EAD allowed for analysis of a wide range of peptide types derived from digested aflibercept in one single DDA. Singularly charged and large sized peptides, PTM localization, and differentiation of amino acid isomers were studied.

MATERIALS AND METHODS

Sample preparation:
Aflibercept was denaturated with 7.2 M guanidine hydrochloride (HCl) in a buffer of 50 mM Tris-HCl, pH 7.0, and free cysteines were capped with 5 mM iodoacetamide. Digestion was performed with either trypsin/Lys-C or trypsin/Lys-C/Asp-N at 30˚ C for 16 h. The reaction was stopped with 1% formic acid.
HPLC conditions:
The digested peptides were separated with a CSH C18 column (1.7 µm particle size, 130 Å, 2.1 × 100 mm, Waters) using an ExionLC 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 300 µL/min. The column temperature was maintained at 50ºC.
MS and MS/MS conditions:
Data were acquired with an information dependent acquisition (IDA) method using the SCIEX ZenoTOF 7600 system.
Data analysis:
Data was processed using the new Biologics Explorer software (SCIEX) with the following nodes:



RESULTS

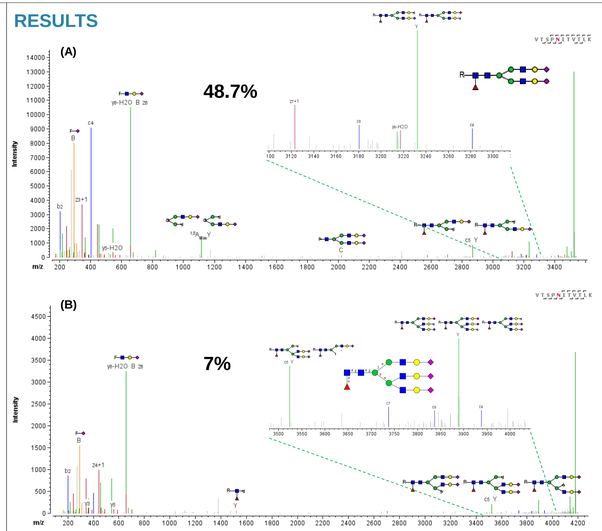


Figure 1. MS/MS EAD spectra of glycosylated peptide VTSPNITVTLK from aflibercept. EAD preserves the fragile glycosylation on the fragment enabling unambiguous localization. The Zeno trap was used to enhance fragment intensity. (A) Most abundant glycopeptide carrying G2F at N36. (B) Peptide carrying G3S3F at N36.

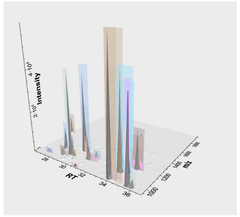


Figure 2. 3D visualization for identified peptides at RT = 27-37 min. X-axis shows RT, y-axis *m/z*, z-axis intensity.

Protein	Location	Modification	EAD_1 [%]	EAD_2 [%]	EAD_3 [%]
Protein	N36	A2G2F1S2	48.7	48.9	49.0
Protein	N36	A2G2F1S1	30.1	29.9	30.7
Protein	N36	A3F1S3	7.3	6.9	6.3
Protein	N36	A3F1S2	6.3	6.2	6.0
Protein	N36	A2G2F1	5.4	5.8	6.0
Protein	N36	A2G1F1S1	0.7	0.7	0.7
Protein	N36	A4F1S3	0.4	0.4	0.4
Protein	N36	A4F1S4	0.2	0.3	0.0
Protein	N36	A3F1	0.2	0.2	0.2
Protein	N36	M4A2G2S1	0.2	0.2	0.2
Protein	N36	Deamidated	0.1	0.1	0.1

Figure 3. Glycans identified at site N36. Three replicates demonstrate the reproducibility of the identification independently of abundance of the glycoform.

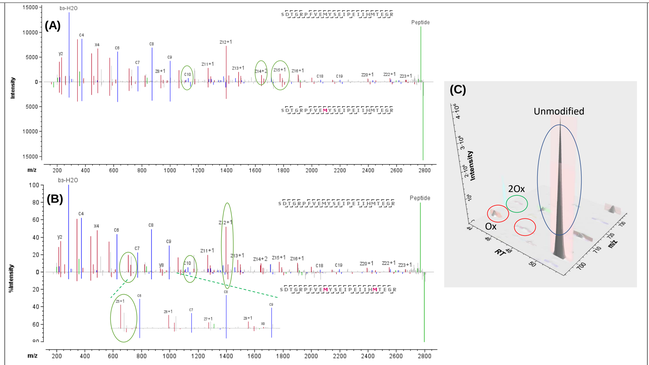


Figure 4. Data for unmodified and oxidized peptide SDTGRPFVE<u>M</u>YSEIPEIIH<u>M</u>TEGR. Rich fragmentation leading to high fragment coverage and confident identification was obtained with the EAD platform method for all peptides shown. Mirror plots of MS/MS EAD spectra for (A) non-modified peptide vs. peptide with one oxidation at M10 and (B) non-modified peptide vs. peptide with two oxidations at M10 and M20. (C) 3D plot for identified peptides visualizes all relevant parameters easily: RT and *m/z* shift for oxidized peptides in comparison to un-modified peptide and intensity difference can quickly be reviewed. Blue circle: unmodified peptide. Red circles: peptide with one oxidation. Green circle: peptide with two oxidations.

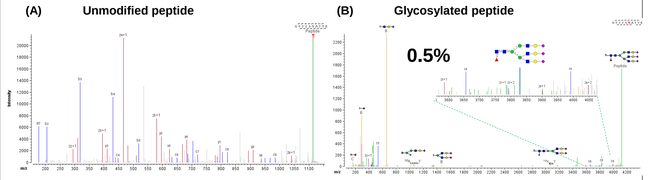


Figure 5. Data for unmodified and glycosylated peptide GFIIS<u>N</u>ATYK. MS/MS EAD spectra of (A) the +1 charged state of the aglycosylated peptide and (B) of the peptide carrying 0.5% G3S3F at N68. Despite the low charge state of +1, rich fragmentation was obtained leading to 89% fragment coverage with EAD for the unglycosylated peptide. EAD is an alternative fragmentation mechanism, which can fragment all charge states of peptides with an easy-to-use platform DDA method. Despite the low abundance of the glycosylated peptide of ~0.5%, fragments with intact glycosylation attached were obtained, providing unambiguous localization information. In addition, peptide backbone information was achieved. The MS/MS data was achieved using an automatically operating ion trap (Zeno trap) prior to the TOF region, which significantly enhances the duty cycle up to 95 %.

Protein	Location	Modification	EAD_1 [%]	EAD_2 [%]	EAD_3 [%]
Protein	N196	A2G2S1	37.8	39.1	39.5
Protein	N196	A2G2	21.7	18.0	19.5
Protein	N196	A2G2S2	15.8	16.2	16.2
Protein	N196	M5	8.3	10.6	8.8
Protein	N196	A2G1	3.0	2.5	2.7
Protein	N196	M4A1G1	2.4	1.6	1.5
Protein	N196	M4A1G1S1	2.2	2.4	2.3
Protein	N196	A2G1S1	2.1	2.4	2.5
Protein	N196	A2G2F1	1.9	1.8	2.0
Protein	N196	M5A1G1S1	1.8	1.9	1.9
Protein	N196	M5A1G1	1.4	1.8	1.5
Protein	N196	M4	0.6	0.4	0.3
Protein	N196	A2G2F1S2	0.3	0.3	0.3
Protein	N196	M4A2G2S1	0.3	0.3	0.3
Protein	N196	M4A2G2	0.3	0.2	0.2
Protein	N196	A2G2Sg1	0.1	0.1	0.1
Protein	N196	A2G1B	0.1	0.1	0.0
Protein	N196	A1	0.1	0.1	0.0
Protein	N196	A2G1F1	0.0	0.1	0.1
Protein	N196	A2G2B	0.0	0.2	0.2

High
Low

Figure 6. Glycans identified on site N196. 20 different glycans were identified on the glycopeptide. Three replicates demonstrate great reproducibility of the identification and relative quantification.

CONCLUSIONS

- MS/MS fragment detection can be significantly enhanced compared to traditional accurate mass MS/MS analysis when using the Zeno trap. This enables confident identification due to excellent MS/MS raw data quality, even for low-abundant species.
- The robust, reproducible and easy-to-use alternative fragmentation technique of EAD enables users to directly answer challenging analytical questions with a platform method on the ZenoTOF 7600 system powered by SCIEX OS software.
- Automatic data analysis with Biologics Explorer software provides an accurate data analysis tool and powerful visualization feature, which enables complex glycan profiling.

REFERENCES

1. Baba T et al. (2015) Electron capture dissociation in a branched radio-frequency ion trap, Anal Chem. 87, 785−792.
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3. Comprehensive peptide mapping of biopharmaceuticals utilizing electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-02-12639-A.
4. A new electron activated dissociation (EAD) approach for comprehensive glycopeptide analysis of therapeutic proteins, SCIEX technical note, RUO-MKT-02-12980-A.

TRADEMARKS/LICENSING