Identification and differentiation of positional isomers of O-linked glycopeptides of etanercept using an alternative electron-based MS/MS approach

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ABSTRACT

This poster highlights the power of electron-activated dissociation (EAD) for the confident identification and unambiguous localization of O-linked glycosylation in etanercept. The unique ability of EAD to pinpoint the positions of glycosylation further enabled the differentiation of positional isomers of O-glycopeptides.

INTRODUCTION

Glycosylation is a common post-translational modification (PTM) that plays a critical role in antibody effector functions.[1] Comprehensive characterization of N- and O-linked glycosylation in protein therapeutics is essential for ensuring drug safety and efficacy. When applied to glycopeptides, traditional collision-based MS/MS approaches, such as collision-induced dissociation (CID), result in the loss of labile glycan moieties. Hence, accurate determination of glycosylation sites using CID is extremely challenging, particularly for O-glycosylation without a consensus sequence. Compared to CID, EAD is superior for glycopeptide analysis, given its ability to preserve the glycan structures in the fragments.[2–4] Etanercept is a dimeric fusion protein that consists of 2 tumor necrosis factor receptor (TNFR)-Fc chains with 3 N-glycosylation and 13 O-glycosylation sites on each chain. In this work, EAD was utilized to elucidate the complex O-glycosylation profile of etanercept on the peptide level (Figure 1).

MATERIALS AND METHODS

Sample:
Etanercept (25 µg/µL) was denatured by 7M guanidine hydrocholoride, reduced with 10mM dithiothreitol and alkylated using 25mM iodoacetamide. The sample solution was buffer-exchanged into 50 mM Tris-HCl (pH = 7.4) using Bio-Spin columns (Bio-Rad), followed by enzymatic digestion at 37°C for 2 h using trypsin (Promega). The resulting solution was incubated with SialEXO from Genovis at 37°C for 4 h to remove sialic acids. A total of 10–20 µL of the final solution (~5–10 µg) was injected for LC-MS analysis.

HPLC:
The peptides were separated using a Waters ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å). A flow rate of 0.25 mL/min was used for the separation. The column was kept at 60°C in the column oven of an ExionLC AD system (SCIEX). The mobile phases A and B consisted of 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile, respectively.

MS/MS:
EAD data-dependent acquisition (DDA) and MRM^{HR} data were acquired in SCIEX OS software using the ZenoTOF 7600 system (SCIEX). EAD MRM^{HR} was specifically applied to O-linked glycopeptides containing 6 or 7 core 1 O-glycans. The EAD DDA and MRM^{HR} data were analyzed using 2 peptide mapping templates within the Biologics Explorer software.

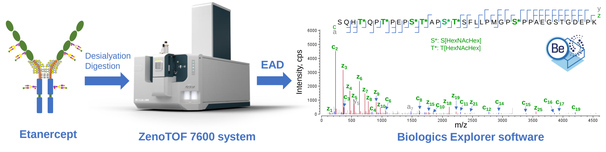

Figure 1. Elucidation of O-linked glycosylation in etanercept using the ZenoTOF 7600 system and Biologics Explorer software from SCIEX. EAD is powerful for the localization of labile glycosylation. The sites of glycosylation in the O-linked glycopeptides of etanercept that contained up to 7 O-linked glycan structures were confidently determined based on the interpretation and annotation of EAD MS/MS spectra using Biologics Explorer software.

RESULTS

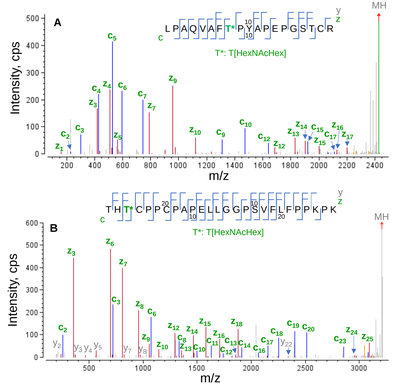

Figure 2. EAD of the singly O-glycosylated peptides, L1-R19 (A) and T243-K268 (B). Two tryptic peptides contained multiple O-glycosylation sites (Ser and Thr) and 1 was modified with the core 1 glycan structure (HexNAcHex). The excellent EAD MS/MS spectra allowed for confident localization of the O-glycan at T8 in peptide L1-R19 (A) and at T245 in peptide T243-K268 (B). Note that all the EAD spectra displayed in this poster are deisotoped spectra taken from Biologics Explorer software. Not all fragments are labeled for spectral clarity.

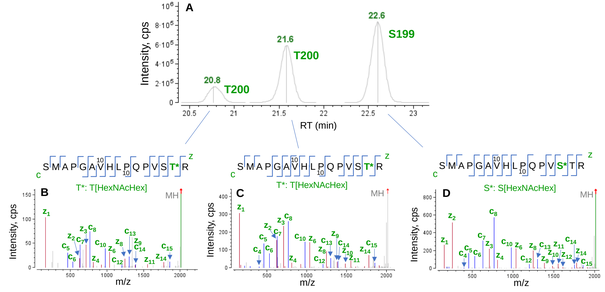

Figure 3. Characterization of 3 isomers of the singly O-glycosylated peptide, SMAPGAVHLPQPVSTR (S186-R201) using EAD (1 eV). Three chromatographic peaks were detected in the extracted ion chromatogram (XIC) of S186-R201 modified with 1 HexNAcHex (A). The high-quality EAD data (B–D) revealed that 2 of the 3 potential O-glycosylation sites in this peptide were occupied (S199 and T200). Based on the m/z of the z_2 and z_3 ions, the peak at RT = 22.6 min (A) was associated with O-glycosylation at S199 (D). Both peaks at RT = 20.8 min and 21.6 min were assigned as the species carrying 1 HexNAcHex at T200 (B and C).

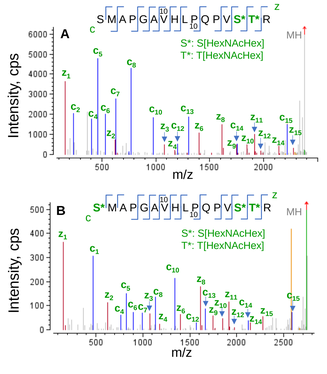

Figure 4. EAD MS/MS spectra (1 eV) of the O-glycopeptide, S186-R201, modified with 2 (A) or 3 (B) HexNAcHex. EAD produced a nearly complete series of c/z fragments for unambiguous localization of multiple HexNAcHex in this O-glycopeptide. For the doubly O-glycosylated species, the detection of a nearly complete series of c/z fragments with or without the glycan enabled accurate localization of 2 HexNAcHex at S199 and T200 near the C-terminus. In the case of the triply glycosylated species (B), the m/z of c series ions confirmed that the Ser residue at the N-terminus was also occupied by HexHAcHex, as expected.

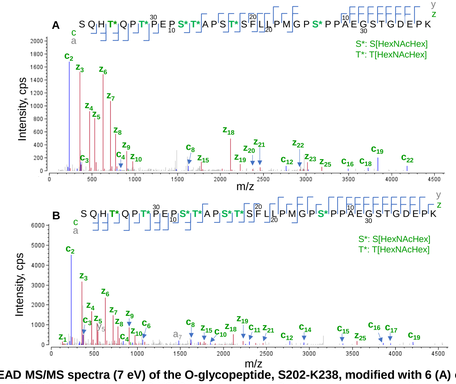

Figure 5. EAD MS/MS spectra (7 eV) of the O-glycopeptide, S202-K238, modified with 6 (A) or 7 (B) HexNAcHex. The O-glycopeptide S202-K308 contains up to 7 O-glycans. Accurate localization of the O-glycans was achieved for the species containing 6 (A) or 7 (B) HexNAcHex using EAD. Note that the EAD MS/MS spectrum is shown for only 1 of 2 positional isomers for the species carrying 6 HexNAcHex. See Figure 6 for the differentiation of the 2 isomers.

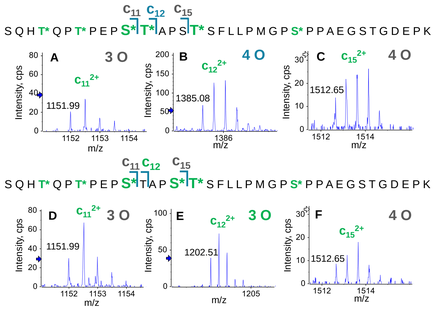

Figure 6. Signature c ions for the differentiation of 2 positional isomers of the O-glycopeptide, S202-K238, containing 6 HexNAcHex. Two isomers (Isomer 1 and Isomer 2) were differentiated based on the detection of c_{11}^{2+} (A and C) and c_{15}^{2+} (C and F) ions at the same m/z and a c_{12}^{2+} fragment containing 4 and 3 HexNAcHex for Isomer 1 (B) and Isomer 2 (E), respectively. The m/z of c_{12}^{2+} indicated that T12 in the sequence was modified with a HexNAcHex in Isomer 1 (B). The m/z of c_{15}^{2+} (F) indicated that S15 was glycosylated in Isomer 2 €.

CONCLUSIONS

- Confident identification and accurate localization of O-glycosylation in etanercept were achieved using EAD
- High-quality EAD data enabled localization of O-glycopeptides containing up to 7 O-glycans.
- Positional isomers of O-glycopeptides were differentiated unambiguously based on a complete or nearly complete series of sequence ions produced in EAD.
- EAD can be leveraged to elucidate complex glycosylation profiles in protein therapeutics with increasing complexity and variety.

REFERENCES

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TRADEMARKS/LICENSING