Characterization of complex glycosylation patterns using a new QTOF mass spectrometer with EAD

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ABSTRACT

Confident comprehensive *O*-linked glycan characterization using electron activated dissociation and an optimized data analysis strategy is presented.

INTRODUCTION

Fetuin is a frequently used MS/MS test molecule with a complex N- and O-glycosylation profile. Comprehensive characterisation of the O-linked glycans is particularly challenging due to the proximity of four of the five previously reported O-glycosylation sites (S271, T280, S282, S296), which in combination with the relative cleavage-sites of standard digestion enzymes, results in a single tryptic peptide of 61 amino acids containing four possible O-glycosylations [1,2].

Electron associated fragmentation is particularly suitable for glycopeptide sequencing because it induces strong cleavage of the peptide backbone, in preference to glycan fragmentation as is commonly observed with CID/HCD [3]. Here we present results acquired using electron activated dissociation (EAD), a form of hot electron capture dissociation, available on a newly developed SCIEX QTOF instrument called the ZenoTOF 7600 system.

With previously described ECD fragmentation, it is typical to observe predominantly c and z• peptide fragments (with some y and z•+H), however the EAD used here also generates b-ions along with NH_3 and H_2O losses. The fragment types considered in the peptide mapping searches were therefore tuned to increase the probability of confident glycan identifications with fewer false positives. Many peptides of interest were relatively large, with charge states up to +8 fragmented, so the workflow was again optimized to consider more than the usual +1 and +2 fragments.

MATERIALS AND METHODS

Sample preparation:
Bovine fetuin from Sigma-Aldrich was proteolyzed using a trypsin/lys-C combination after reduction and carbamidomethylation of disulphide bonds.

HPLC conditions:
Peptides were separated using reverse-phase LC with an ExionLC UHPLC system over a gradient length of 45 minutes. Samples were injected in triplicate with 1, 5, 10 and 20 pmol injected on column..

MS/MS conditions:
MS and MS/MS data were acquired using a new SCIEX ZenoTOF 7600 system using EAD fragmentation in a data-dependent mode with up to ten MS/MS events per cycle and an accumulation time of 100 ms. The Zeno trap allows for a considerable increase in sensitivity (up to 20x for low m/z ions) compared to previous generation instruments [4].

Data processing:
Data were processed using a new stand-alone desktop version of the Genedata Expressionist software ('Biologics Explorer') for biotherapeutic characterization. This software has specifically optimized workflows for analysis of biologics using data acquired with SCIEX QTOF mass spectrometers for both CID and (as here) EAD fragmentation (Figure 1).

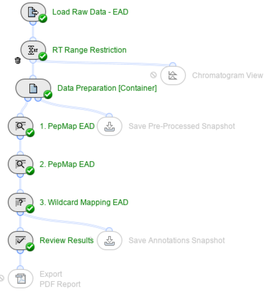

Figure 1. Extended peptide mapping data analysis workflow from Biologics Explorer. The combination of workflow activity nodes have been optimized to extract high confidence meaningful results for deep characterization of biotherapeutics.

The first steps in the pipeline are loading and processing of the raw LC-MS data. Peaks are found in the MS1 'survey' and clustered (isotopes, charge states and adducts) using parameters optimised for the ZenoTOF 7600 system data. Dependent MS/MS are then processed and associated with the MS1 precursors.

Data are then processed automatically using a 'waterfall' strategy that parses the search space into sequential steps:

- The first search activity (1. PepMap) aims to identify MS/MS spectra corresponding to the most probable or expected peptides, for example those with a limited number of missed cleavages, or as in this case, the well characterised *N*-linked glycans.
- Confidently identified spectra are then removed from consideration ('*Ignore Annotated Features*')
- The remaining unidentified spectra are searched again in the second search activity (2. PepMap) using more refined criteria, and a semi-tryptic digestion to identify the remaining modifications, here specifically the *O*-linked glycans.
- The third search activity (3. Wildcard Mapping) enables the identification of unexpected modifications present in the sample, such as over-alkylation.

The consequence is a considerable reduction in the false positive rate.

RESULTS

Data acquired in EAD-mode using a new QTOF mass spectrometer was successfully loaded and imported into Biologics Explorer. Raw data can be viewed and visually assessed using an ion map or traditional total ion chromatogram (TIC) / base peak chromatogram (BPC) display (Figure 2).

(a) (b)

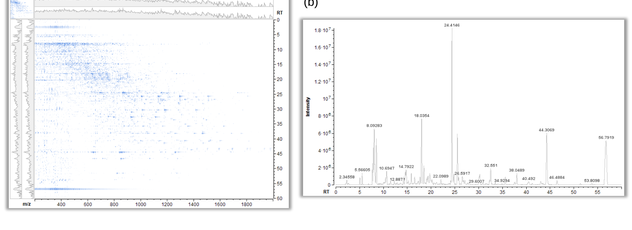

Figure 2. Data visualization of fetuin tryptic digest in Biologics Explorer. (a) Two-dimensional ion map display of data. (b) Traditional TIC display.

The EAD fragmentation and search strategy enabled confident, positive identification of *O*-linked glycans (Figure 3). The EAD fragmentation does not fragment the glycan itself as with other fragmentation types, such as CID, and as such it is possible to confidently confirm the glycan and its location on the peptide backbone.

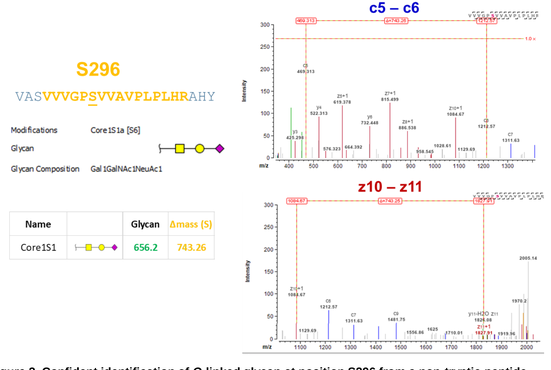

Figure 3. Confident identification of *O*-linked glycan at position S296 from a non-tryptic peptide. The mass of the *O*-linked glycan Core1S1, plus the mass of the serine to which it is attached, gives a mass shift of 743.26 Da between the associated fragment ions in the EAD MS/MS spectra; this can be clearly seen (using the measuring tool in Biologics Explorer). The positions with observed fragments are marked in the annotation in the upper-right corners showing that nearly complete sequence coverage was observed.

	EAD	CID
Fetuin A coverage	98.2%	100%
Fetuin B coverage	77%	93%
N-glycans	N81 N138 N158	N81 N138 N158
O-glycans		
Core1S1	S253 T262 S264 S278	S253 T316
Core1S2	S253 T262	
Core2ExtS2	S253 T262	S253 T262
Phosphorylation	S120 S302 S305 S306 S307 T316	S120 S302 S305 S306 S307 T316
Num. Proteins	23	23

Table 1. Comparison of fetuin analysis using EAD and CID fragmentation with the same ZenoTOF 7600 system.

The same sample of fetuin was also analyzed using CID fragmentation to enable a comparison. The higher sequence coverage with CID is most likely a product of the more efficient dissociation achieved using this fragmentation technique, i.e. more of the parent ion is fragmented. With EAD fragmentation, much of the parent ion remains intact, so higher sample load may be necessary to achieve optimal sequence coverage.

The key difference between the EAD- and CID-based analyses was the successful identification of *O*-linked glycans and the ability to clearly define their position within the peptide. Eight of the previously identified [1,2] *O*-linked glycans were confidently found.

This analysis also brought to light the level of impurity within the Sigma-Aldrich fetuin sample, with over 20 additional bovine proteins identified (Table 1).

Protein	MW [Da]	Abundance [mol/mol]	Amount [w/w]	Amount [w/w]**
A1AT	43694	0.14%	0.14%	0.14%
A1BG	51285	0.33%	0.39%	0.41%
A2MG	165053	1.10%	4.21%	4.42%
ActinB	41606	0.02%	0.02%	0.02%
Adiponectin	24391	0.10%	0.05%	0.06%
Albumin	66433	0.09%	0.13%	0.14%
AMBP	37293	0.10%	0.08%	0.09%
Angiotensinogen	49230	0.13%	0.14%	0.15%
APOA1	28432	0.03%	0.02%	0.02%
APOH	36295	0.05%	0.04%	0.04%
CO3	185047	0.10%	0.42%	0.44%
Fetuin-A*	**41328**	**91.40%**	**87.19%**	**91.57%**
Fetuin-B*	43647	0.97%	0.98%	1.03%
HBBF	15859	0.03%	0.01%	0.01%
ITIH1	71584	0.02%	0.03%	0.03%
ITIH3	68958	0.06%	0.09%	0.10%
ITIH4	98686	0.32%	0.73%	0.77%
KNG1	46383	0.27%	0.28%	0.30%
Plasminogen	88393	0.08%	0.16%	0.17%
PROS	70564	0.01%	0.02%	0.02%
VTDB	51532	0.07%	0.08%	0.08%
rLys-C	48213	4.11%	4.57%	-
TRY	18759	0.49%	0.21%	-

Table 1. Identified impurities in fetuin sample. The table shows that a large number (19) of bovine impurity proteins were also confidently identified in the sample. (This is in addition to fetuin itself and the expected digestion enzymes LysC and trypsin). In one case the level was over 1% by mole or 4% by weight.

CONCLUSIONS

The combination of the ZenoTOF 7600 system and the Biologics Explorer software can perform comprehensive characterisation of biologics. The results for the particularly challenging case of fetuin *O*-linked glycans demonstrate this capability.

In this work the algorithm used to score a putative sequence against an MS/MS spectrum did not consider actual glycan fragments themselves. As mentioned, EAD fragmentation predominantly induces cleavage of the peptide backbone rather than the glycan, so this was not a significant limitation. Nonetheless future work may also consider these fragments.

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TRADEMARKS/LICENSING