Comprehensive Analysis of IgE Glycoforms by FAIMS-LC-MS/MS using Orbitrap Eclipse Mass Spectrometer

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ABSTRACT

Purpose: Correctly and confidently identify N-glycosylation sites and its glycoforms in a biologic using High-Field Asymmetric Waveform Ion Mobility Spectrometry System (FAIMS Pro) interfaced to the Orbitrap Eclipse Mass Spectrometer.

Methods: LC/MS analysis was performed on Thermo Scientific™ Orbitrap Eclipse™ platform coupled with Thermo Scientific™ FAIMS Pro™ interface. The raw files were analyzed using Thermo Scientific™ Proteome Discoverer™ 2.4 software with Byonic node, and Byologic®, or Thermo Scientific™ BioPharma Finder™ 4.0 software.

Results: We successfully identified 7 glycosylation sites including N383 in IgE. Using HCD or ETD fragmentation in combination with optimized FAIMS CV settings we were able to identify significantly more different glycoforms than without FAIMS. Overall, the FAIMS-LC-MS/MS workflow significantly improves glycoform profiling and characterization in heavily glycosylated proteins without enrichment or pre-fractionation.

INTRODUCTION

Liquid Chromatography coupled with high resolution mass spectrometry has proven to be an indispensable analytical tool for identification, characterization and quantification of glycoproteins in complex biological samples and various biologics. Accurate glycoform profiling and identification of a glycosylation site could be difficult due to microheterogeneity present at the glycosylation site, which could get further complicated if the protein carries multiple glycosylation sites in close proximity within the same proteolytic digestion product. In this study, we present an application to correctly and confidently identify N-glycosylation sites and its glycoforms of IgE (Figure 1) using High-Field symmetric Waveform Ion Mobility System (FAIMS Pro) interfaced to the Orbitrap Eclipse Mass Spectrometer. FAIMS separates gas-phase ions on the basis of differences in their ion mobility in high and low electric fields[1]. It has previously been demonstrated that FAIMS improves dynamic range, increases signal-to-noise and reduces interference from ions of similar *m/z* [1-2]. The study also utilizes the Tribrid MS platform assisted by multiple fragmentation techniques to efficiently and correctly characterize multiple glycosylation sites.

MATERIALS AND METHODS

Sample Preparation

Human Immunoglobulin epsilon heavy chain (IgE, Uniprot accession P01854,) obtained from Millipore Sigma was reduced, alkylated and digested by a combination of LysC/Trypsin with/out Chymotrypsin.

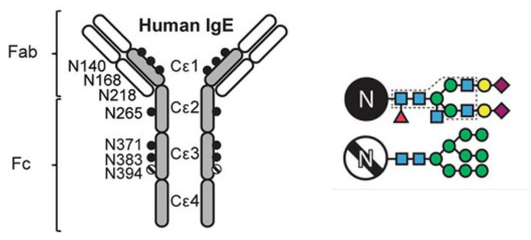
Liquid Chromatography and Mass Spectrometry

Samples were separated by RP-HPLC using a Thermo Scientific EASY-nLC™ 1200 UPLC system connected to a Thermo Scientific™ EASY-Spray™ column, 50 cm × 75 µm over a 120 min 5-40%,3 min from 40-98 % gradient (A: water, 0.1% formic acid; B: 80 % acetonitrile, 0.1% formic acid) at 300 nL/min flow rate. The samples were analyzed on the Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer with or without FAIMS Pro device using DDA FT HCD, EThcD MS2 or HCDpdEThcD methods. FT MS1 was acquired at resolution settings of 60–120K at *m/z* 200 and FTMS2 at resolution of 30–60K at *m/z* 200.

Data Analysis

The Thermo Scientific™ ProSightPC 4.1, BioPharma Finder 4.0 software, and Proteome Discoverer™ 2.4 software with the Byonic™ search node and Byologic (Protein Metrics) were used for glycopeptide data analysis and glycoform quantification. Data were searched against a database containing the Uniprot/SwissProt entries of the model proteins with/out common contaminants and 57 human plasma glycans with a 1% FDR criteria for protein spectral matches. The peptide spectra were also manually validated to confirm identification of multiple glycosylation site.

Figure 1. Schematics of IgE structure and predicted N-glycosylation sites in each constant domain

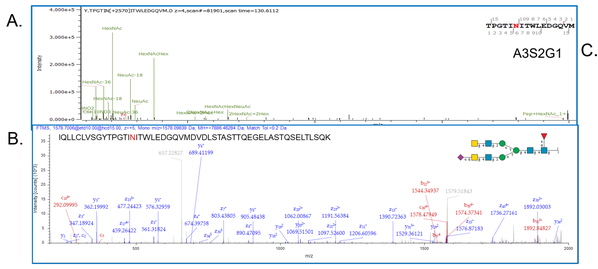


RESULTS

IgE glycosylation sites identification.

Human IgE has seven N-linked glycosylation sites distributed across the constant heavy chains and plays a major role for initiation of allergy reactions. IgE glycosylation play a key role in allergic inflammation and has subject of extensive interest [3-6]. To study IgE glycosylation mostly two types of proteolytic digestion are utilized: chymotrypsin and trypsin. The chymotrypsin digestion resulted in formation of multiple small peptides which are more favorable to HCD fragmentation technique and produced in some cases mostly glycosidic fragments (Figure 2A). The later complicates direct identification of glycosite or relative glycoform profiling. However, glycopeptides with longer peptide sequences created in LysC/tryptic digests carried higher charges and can be analyzed by ETD which produces mostly peptide backbone information (Figure 2B,3B). ETD spectra enabled direct glycosite identification and confirmation even for peptides with multiple sites. All seven IgE N-glycosites were confirmed by MS2 spectra.

Figure 2. Identification of IgE *N*-265 glycopeptide in chymotrypsin digest by Byonic(A) or in trypsin digest by Byonic node in Proteome Discoverer 2.4 software(B). All identified glycoforms for N265 site (C).



C.

Gn	A2S1G1F
M2	A2S2F
M3	A2S1G1
M5	A2G0
M6	A3S1G2F
M9	A3S1G2
A1S1	A3
A2G2F	A2G2B
A2S1G1FB	A2G2FB
A2S2	A3S1G2
A2G1	A3S2G1
A2G1F	A1S1

Evaluation of FAIMS for N- glycopeptide analysis.

The identification of glycopeptides by LC-MS/MS presents significant analytical challenges due to their low abundance and higher charge state distribution compared to tryptic peptides. We evaluated the use of a FAIMS device for N-linked glycopeptide analysis. Different compensation voltages (CV) between -30 and -60V were tested with 5-10V resolution using 2 approaches: external (inter-analysis CV switching) and internal stepping (intra-analysis CV switching).

Figure 3. N383 Site Glycosylation Profile Analysis: HCD (A) and EThcD (B) Identification spectra(A) and Number of Identified glycoPSMs with/out FAIMS(C)

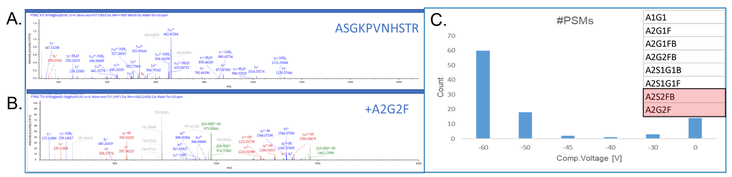
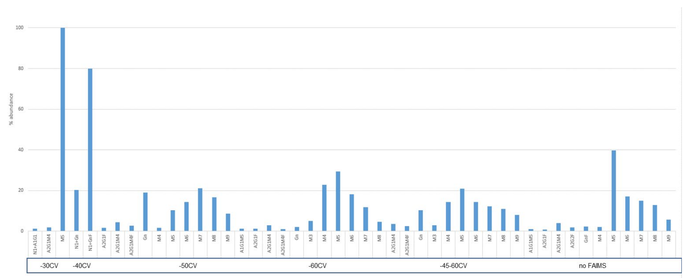


Figure 4. Relative Abundances of N394 Glycoforms in LysC/Trypsin Digest at different CVs



As shown in Figures 3 & 4 more PSMs and unique glycoforms were identified using -45-60CVs in both cases for partially glycosylated N383 site and functionally most important site N394. At CV -30-40CVs we identified mostly unglycosylated peptides. We did observe some sialic acid lost at CV less than -60. As ionization efficiency of glycopeptides depends upon both peptide backbone and glycan composition we did not detect a clear trend of CV value vs glycan composition (Table 1) in CVs ranges from -40 to -60. However we did observe improved S/N (dynamic range) in all FAIMS spectra which can explain higher number of identified glycopeptides especially for partially glycosylated sites as N383 and detection some very low abundance glycoforms (Figure 2A,B).

Table 1. Most Abundant IgE glycoforms identified per site at different CV. Each glycoform was calculated as a sum of all detected peptides including sodiated species.

	No FAIMS	-30CV	-40CV	-50CV	-60CV	-45/-60CV
N140	A2S1G1F	nonglycos	A2S2F	A2S2FB	A2G0F	A2G0F
N168	A2S1G1B	nonglycos	A2S2B	A2S1G1	A2G1	A2G1
N218	A2S2F	nonglycos	A2S2F	A2S2F	A2S1G1F	A2S2F
N265	A2G2F	nonglycos	A2S2	A2S2	A2S1G1FB	A2S2
N371	A2S1G1FB	A2S1G1	A2S2FB	A2S1G1FB	A2S1G0FB	A2S1G1FB
N383	A2G2F	A2G2F	A2S2FB	A2G2F	A2G2F	A2G2F
N394	M5	M5	GnF	M7	M5	M5

CONCLUSIONS

- All seven IgE N-linked glycosites in both non and glycosylated states were identified by MS2 experiments.
- The primary N-linked glycoforms of 394 site are oligomannose species as expected however complex biantennary glycans were observed as well.
- In all experiments, internal CV stepping generated best identification rates vs. external CV stepping or single CVs and 45-60 2CV method provided most identifications.
- FAIMS-LC-MS/MS workflow significantly improves glycoform profiling and characterization in heavily glycosylated proteins without enrichment or pre-fractionation

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TRADEMARKS/LICENSING

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