Confident and Efficient Characterization of Recombinant Human Follicle Stimulating Hormone with the Thermo Scientific Q Exactive HF-X BioPharma Platform

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ABSTRACT

Purpose: To demonstrate comprehensive characterization of recombinant human follicle stimulating hormone (rhFSH) on a hybrid orbitrap platform.

Methods: Intact and peptide separations were performed using a Thermo Scientific™ Vanquish™ UHPLC System coupled to a Thermo Scientific™ Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, while released N-glycan analysis used a Thermo Scientific™ Ultimate™3000 HPLC System. Data analysis was performed using Thermo Scientific™ BioPharma Finder™ Software.

Results: Intact mass analysis reveals 117 alpha chain glycoforms and 47 beta chain glycoforms. Peptide mapping of rhFSH achieved 100% sequence coverage and revealed the site and abundance of a number of key post-translational modifications. For N-glycan analysis, a total of 46 reported glycans were identified, including both neutral and sialic acid variants.

INTRODUCTION

There is a continued focus within the biopharmaceutical industry on the development of biotherapeutic products for the treatment of a variety of diseases and conditions. Regulatory bodies require complete characterization, including a full assessment of post-translational modifications. Recombinant human follicle stimulating hormone (rhFSH) is an approved biotherapeutic for the treatment of infertility and several branded products exist on the market today. FSH is a heterodimeric protein, containing alpha and beta subunits bound together by a complex disulphide bridge arrangement. FSH also contains many silaylated glycans, which are the primary driver of biological activity *in vivo*. Comprehensive characterization of rhFSH post-translational modifications is demonstrated using the Thermo Scientific Q Exactive HF-X Mass Spectrometer.

MATERIALS AND METHODS

Sample Preparation

For peptide mapping and disulphide assignment, reduced, non-reduced and alkylated rhFSH was digested with trypsin or trypsin combined chymotrypsin(disulphide assignment only). For released N-glycan analysis, rhFSH was deglycosylated by PNGase F and labelled using 2-AB.

Method

Intact mass analysis was performed using a Thermo Scientific™ MAbPac™ RP (2.1mm x 50 mm, 4 µm) column over a 4.8 min gradient. For MS parameters, 240,000 resolution and 3 microscans were used.

For peptide mapping and disulphide assignment, reduced, non-reduced and alkylated rhFSH was digested with trypsin and analyzed using a Thermo Scientific™ Accucore™ Vanquish™ C18+ (2.1mm x 150 mm, 1.5 µm) column over a multi-step 50 min gradient (4-40% ACN). Top 10 method was chosen, 60,000 resolution for MS1 and 15,000 resolution for MS2. All intact protein and peptide separations were performed using a Thermo Scientific Vanquish UHPLC System coupled to a Q Exactive HF-X mass spectrometer.

Released N-glycan analysis was performed following PNGase F deglycosylation and 2-AB labelling, using a Thermo Scientific™ GlycanPac™ AXH-1 (2.1 x 150 mm, 1.9 µm) column. All glycan separations were performed using a Thermo Scientific UltiMate 3000 HPLC System coupled to a Q Exactive HF-X mass spectrometer. Both fluorescence (excitation spectrum 330nm, emission spectrum 420nm) and MS (negative mode, 120,000 resolution) were used for detection.

Data Analysis

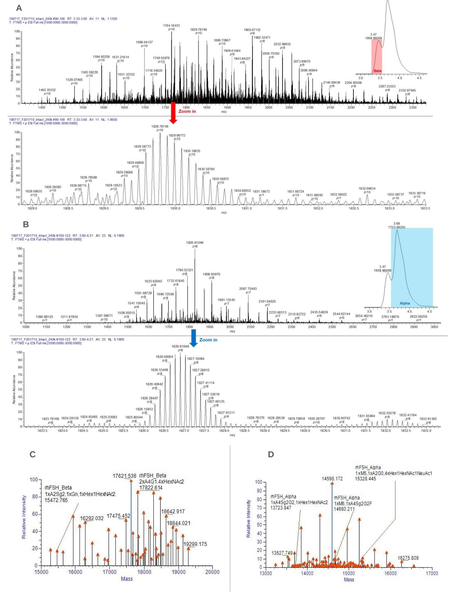
Data analysis was performed using Thermo Scientific BioPharma Finder Software.

RESULTS

Intact Analysis

FSH is a heterodimeric protein, containing alpha and beta subunits bound together by a complex disulphide bridge arrangement. FSH also contains many silaylated glycans, both N- and O-glycans were reported[1]. Two chromatography peaks were observed on RPLC, representing beta and alpha subunits respectively as shown below.

Figure 1. MS spectra of two subunits and deconvolution results. A, MS spectrum of beta subunit. B, MS spectrum of alpha subunit. C, deconvolution result of beta subunit. D, deconvolution result of alpha subunit.



Using 240,000 mass resolution setting on the Orbitrap instrument, the monoisotopic mass for these species can easily be measured. For beta chain shown in figure 1A and 1C, due to the heterogeneity glycans composition, 47 components spread from 15.3-19.3kDa were detected. For all components of beta chain, the delta mass between theoretical and measured mass are less than 0.16Da. Even for component only takes 0.43% of the whole peak, the matched mass error is 2.8ppm(0.051Da) benefits from the high resolution, accuracy and sensitivity of the Orbitrap analyzer. For alpha chain shown in figure 1B and 1D, 117 components spread from 13.2-16.6kDa were detected. For all components of alpha chain, the delta mass between theoretical and measured mass are less than 0.11Da. Even for the component constituting only 0.10% of the whole peak, the matched mass error is 3.6ppm (0.051Da).

Peptide Mapping

In the peptide mapping experiment, reduced and alkylated rhFSH was digested with trypsin and 100% sequence coverage was achieved (figure 2). Multiple key post-translational modification sites and abundance information were revealed, such as deamidation and oxidation(figure 3,4 and table 1).

Figure 2. Peptide mapping results of reduced and alkylated rhFSH.

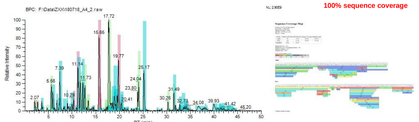


Table 1. Key Modification summary of reduced and alkylated rhFSH.

Site	Modification	Abundance
Alpha M29	Oxidation	2.80%
Alpha M47	Oxidation	5.13%
Alpha M71	Oxidation	4.26%
Beta M109	Oxidation	3.76%
Alpha N66	Deamidation	11.21%
Beta Q81	Deamidation	74.89%

Figure 3. MS/MS spectra comparison between oxidized and naked peptides of beta M109(3.76%).

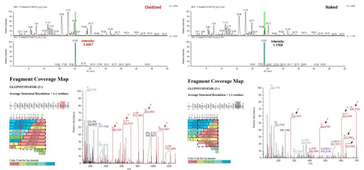


Figure 4. MS/MS spectra comparison between deamidated and naked peptides of alpha N66(11.21%).

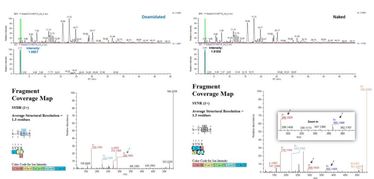


Figure 3 and Figure 4 showed MS/MS spectra of peptides with key modified sites, such as oxidation and deamidation. Arrows indicate fragment ions which could prove modifications. With high quality MS/MS spectra, not only series of b/y ions could be detected, but even the y_2 ions with a mass shift of 0.9837Da between deamidated and naked peptides were identified (figure 4), providing solid evidence of asparagine deamidation.

For disulphide assignment, non-reduced rhFSH was alkylated with IAM first, followed by trypsin digestion or trypsin combined chymotrypsin. Because of the high complexity of disulphide composition, as shown in table 2, the majority disulphide linkages were identified in scramble form even with IAM treatment before digestion.

Table 2. Disphide linkage summary. T+CT, trypsin combined chymotrypsin digestion.

Subunit								
Alpha	Site	C28	C7-C10	C59,C60/C28,C31,C32/C82,84,87				
	Disphide	Free Cys	Scramble	Scramble (MS1 only)				
	Digestion	T+CT	T+CT	T+CT				
Beta	Site	C51/C66/C104	C3	C87-C94	C3-C51	C17-C104	C17-C20	C28-C20
	Disphide	Free Cys	Free Cys	Correct	Correct (MS1 only)	Scramble	Scramble	Scramble
	Digestion	Trypsin	T+CT	Trypsin	T+CT	T+CT	T+CT	T+CT
	Site	C51-C104	C28-C32	C104-C66	C104-C32	C28-C104	C82-C84/C87-C94	
	Disphide	Scramble	Scramble	Scramble	Scramble	Scramble	Scramble	
	Digestion	T+CT	T+CT	T+CT	T+CT	T+CT	T+CT	

N-Glycan Analysis

rhFSH glycosylation exhibits both macro and microheterogeneity[2,3]. It was reported that alpha subunit have two N-glycosylation sites (N52 and N78) and beta subunit also have two modification sites (N7 and N24)[3]. Because the glycans are highly silaylated, a Thermo Scientific GlycanPac AXH-1 (2.1 x 150 mm, 1.9 µm) column was used for glycans separation based on the number of sialic acids(figure 5).

Figure 5. Chromatogram of 2-AB labeled released N-glycans, detected by FLD.

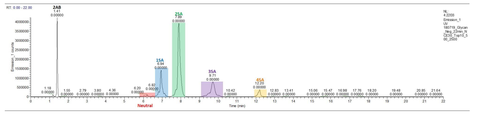


Figure 6. MS spectra of typical N-glycans with different numbers of sialic acids. A, neutral. B, one sialic acid. C, two sialic acids. D, three sialic acids. E, four sialic acids. SA means sialic acid.

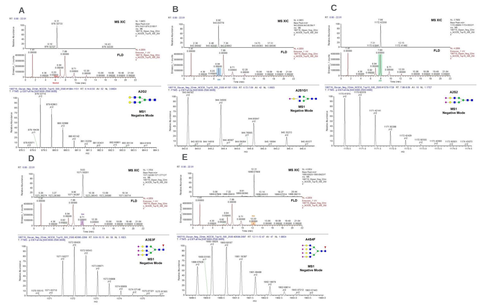


Figure 6 showed the accurate mass and isotopic envelope distribution of typical N-glycans with different number of sialic acids. The high-resolution Orbitrap mass spectrometer gave us the exact mass of the glycans, which helped us to confirm against data in published papers[2,3], as shown in figure 7.

Table 3 showed some high abundance N-glycopeptides identified in peptide mapping experiment. It is easy to determine that the predominant type is two sialic acids contained N-glycans; at the same time three or four sialic acids contained types were identified, which was consistent with released glycans analysis.

Figure 7. 46 identified N-glycans which have been reported.

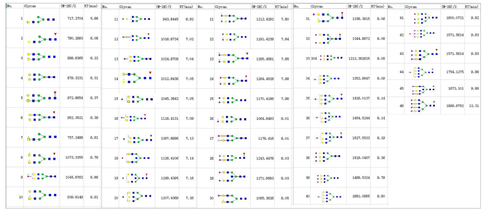


Table 3. High abundance N-glycopeptides identified in peptide mapping.

Site	N-glycans
Alpha N52	A2G2/A2S2/A2S2Ac/A2S2F/A2S2G1/A2S1G0/A2S2G1/A2S1G1F/A3S3
Alpha N78	A2S2/A2S2F/A2S2G1/A3S3
Beta N7	A4G4/A4S4/A4S4F/A3S3F/A4S3G0F/A2S1G1F/A3S3
Beta N24	A2S2F

CONCLUSIONS

- Intact mass analysis reveals 117 alpha chain glycoforms and 47 beta chain glycoforms using 240,000 resolution.
- Peptide mapping of rhFSH achieved 100% sequence coverage and revealed the site and abundance of a number of key post-translational modifications.
- For N-glycan analysis, a total of 46 reported glycans were identified, including both neutral and sialic acid variants.
- Confident and efficient characterization of the complex glycoprotein rhFSH on an advanced Orbitrap platform.

REFERENCES

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3. George R. Bousfield, Jeffrey V. May, John S. Davis, James A. Dias. In Vivo and In Vitro Impact of Carbohydrate Variation on Human Follicle-Stimulating Hormone Function. Front. Endocrinol. 9:216.doi: 10.3389/fendo.2018.00216

TRADEMARKS/LICENSING

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