High-sensitivity Host Cell Protein (HCP) analysis on a new Orbitrap Excedion Pro BioPharma hybrid mass spectrometer with optimized sample preparation

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Abstract

Purpose: To demonstrate the capability of a new Thermo Scientific™ Orbitrap™ Excedion™ Pro BioPharma mass spectrometer for HCP profiling and compare results using different sample preparation protocols.

Methods: LC-MS/MS based peptide mapping was employed for NISTmAb HCP profiling using native digestion, HCP of trastuzumab that had undergone affinity column purification using native digestion and Protein A depleted Thermo Scientific™ EasyPep™ digestion were compared.

Results: Demonstrated the ultrahigh-sensitivity HCP profiling with a new Orbitrap Excedion Pro BioPharma mass spectrometer and compared the trastuzumab HCP results using two different digestion approaches.

Introduction

Recombinant biotherapeutics are synthesized using non-human host cell lines. Residual host cell proteins (HCPs) can potentially impact the safety and efficacy of the final drug product if their levels exceed certain limits. While ELISA is typically employed to quantify the total amount of HCPs, it cannot address the differential immunogenic relevance and impact on product stability of individual HCPs. LC-MS has emerged as a complementary method, enabling the identification and monitoring of individual HCPs. However, the intrasample dynamic range (usually greater than six orders of magnitude) between HCPs and the drug product presents a significant challenge in detecting HCPs present at very low levels in biotherapeutics. Here, we showcase the performance of the new Orbitrap Excedion Pro BioPharma hybrid mass spectrometer for highly sensitive HCP analysis using NISTmAb. Additionally, we evaluated different sample preparation methods for HCP analysis of trastuzumab samples.

Materials and methods

Sample preparation

NISTmAb sample preparation:

Diluted to 2 mg/mL in digestion buffer and digested with trypsin under native conditions as optimized in a previous publication[1].

Trastuzumab (Affinity Column Purified, Figure 1) sample preparation:

1mg sample for each digestion, respectively. Native digestion was following the same protocol as NISTmAb. The Protein A depleted EasyPep™ digestion used the protocol Jae et.al. reported before[2] as shown in Figure 2.

UHPLC-HRAM-MS methods

A Thermo Scientific™ Acclaim™ Vanquish™ C18 UHPLC column, 2.1x250mm (P/N 074812-V) was used for peptide separation at 300 µL/min flow rate over a 135 min step gradient. A new Orbitrap Excedion Pro BioPharma mass spectrometer was used. MS settings listed in Table1.

Data analysis

Data analysis was performed using Thermo Scientific™ Proteome Discoverer™ 3.2 software with the CHIMERYS™ intelligent search algorithm, utilizing the UniProt Mus musculus database for NISTmAb and the UniProt Cricetulus griseus database for trastuzumab.

Figure 1. Trastuzumab expressed in CHO cell line that had undergone affinity column purification was used for sample preparation workflows evaluation.

Figure 2. Protein A depleted Thermo Scientific™ EasyPep™ digestion workflow.

Results

HCP analysis of NISTmAb using native digestion

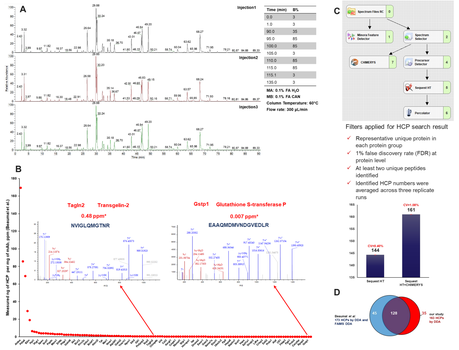
Figure 3A displayed the Base Peak Chromatogram (BPC) profiles of three replicate injections of NISTmAb using native digestion. For each injection, 80 µL (≈176 µg NISTmAb was loaded onto the column, if digestion was complete. During this study, 163 HCPs were identified in NISTmAb using <1% peptide and protein FDR confidence and at least 2 unique peptides per protein filters, in all three replicate injections.

HCPs spanning the abundance range which was published previously [1, 3] were identified from >100 ppm (fructose-bisphosphate aldolase A) to 0.007 ppm (Glutathione S-transferase P, Table2). For peptide associated to low abundant HCPs, MS2 spectra with high S/N still can be acquired for identification (Figure 3B), indicated the excellent sensitivity of the new Orbitrap Excedion Pro BioPharma mass spectrometer, and the wide dynamic range of this instrument can facilitate the identification of proteins distribute from high to low concentrations.

Both Sequest® HT and CHIMERYS intelligent search algorithms were used to search DDA MS2 spectra using UniProt Mus musculus database. The combination of two searching engines can provide more identifications (Figure 3C), and searching results were filtered using <1% peptide and protein FDR confidence and at least 2 unique peptides per protein filters. The CV of identified HCPs among three replicate injections was 1.08%, which proved excellent reproducibility and stability of this workflow.

We also compared our results with Beaumal *et.al.* reported [3], 128 out of 163 HCPs from our study were also identified in previous publication, which employed FAIMS DDA + DDA MS2, and 173 HCPs were identified (Figure 3D). 31 HCPs from our study were newly detected compared to the results published by Beaumal *et.al* (Table 3). These HCPs include some enzymes that may affect product stability, indicated the necessity of using different method/platform for potential high-risk HCP identification.

Figure 3. HCP results of NISTmAb treated with native digestion. A, BPC profiles of three injection replicates. B, MS2 spectra of peptides associated to two low abundant HCPs, Transgelin-2 (0.48 ppm, left panel) and Glutathione S-transferase P (0.007 ppm, right panel). C, database searching workflow using CHIMERYS + Sequest HT search algorithms. Filters at protein/peptide levels were applied. The combination of CHIMERYS + Sequest HT gave more results than Sequest HT only. D, The HCPs identified in this study correlate well with recently published results by Beaumal et al. [3], and more unique HCPs were identified using our approach.



Filters applied for HCP search result

- Representative unique protein in each protein group
- 1% false discovery rate (FDR) at protein level
- At least two unique peptides identified
- Identified HCP numbers were averaged across three replicate runs

Table 1. MS settings.

Source parameters	
Spray Voltage (V)	+3500
Sheath Gas (Arb)	40
Aux Gas (Arb)	10
Ion Transfer Tube Temp (°C)	325
Vaporizer Temp (°C)	250
Application Mode	Peptide
Full MS	
Orbitrap Instrument Resolution	120,000
Scan Range (m/z)	350-1200
Maximum Injection Time (ms)	50
AGC Target	Custom
Normalized AGC Target (%)	300
RF Lens(%)	40
Include charge states	2-6
ddMS2	
Number of Dependent Scans	15
Isolation Window (m/z)	2
Activation Type	HCD
HCD Collision Energies (%)	30
Orbitrap Resolution	30,000
First Mass (m/z)	120
Maximum Injection Time (ms)	150
AGC Target	Custom
Normalized AGC Target (%)	100

Table 2. Subset of 20 HCPs identified in this study matched against measured ng of HCP per mg of mAb shown by Beaumal *et al.*

Gene Name	Description	Measured ng of HCP per mg of mAb, ppm (Beaumal et al.)
Aldoa	Fructose-bisphosphate aldolase A	169.387
Aldoc	Fructose-bisphosphate aldolase C	85.843
Pdia6	Protein disulfide-isomerase A6	69.000
Gpi	Glucose-6-phosphate isomerase	29.354
B2m	Beta-2-microglobulin	19.049
Abhd11	sn-1-specific diacylglycerol lipase ABHD11	6.467
Ppt1	Palmitoyl-protein thioesterase 1	5.735
Grn	Progranulin	5.261
Ptgr1	Prostaglandin reductase 1	4.376
Sema4b	Semaphorin-4B	4.075
Spen	Msx2-interacting protein	4.071
Sf3a1	Splicing factor 3A subunit 1	3.968
Srsf7	Serine/arginine-rich splicing factor 7	3.670
Thrap3	Thyroid hormone receptor-associated protein 3	3.631
Papln	Papilin	3.589
Nsf1c	NSFL1 cofactor p47	3.118
Mars1	Methionine--tRNA ligase, cytoplasmic	3.071
Hnrnpa2b1	Heterogeneous nuclear ribonucleoproteins A2/B1	3.043
Eif4b	Eukaryotic translation initiation factor 4B	2.978
Clint1	Clathrin interactor 1	2.407

Table 3. HCPs identified in this study and not reported by Beaumal *et al.*

Gene Name	Description	# Unique Peptides	# PSMs
Nherf1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	6	26
G3bp1	Ras GTPase-activating protein-binding protein 1	5	51
Cnbp	CCHC-type zinc finger nucleic acid binding protein	4	33
Eny2	Transcription and mRNA export factor ENY2	4	35
Hnrnph1	Heterogeneous nuclear ribonucleoprotein H	4	31
Pafah1b3	Platelet-activating factor acetylhydrolase IB subunit alpha1	4	22
Adh5	Alcohol dehydrogenase class-3	3	10
Cfp	Properdin	3	18
Clib	Isoform 2 of Clathrin light chain B	3	14
Lman2l	VIP36-like protein	3	10
Man2b1	Lysosomal alpha-mannosidase	3	6
Mt2	Metallothionein-2	3	10
Myef2	Myelin expression factor 2	3	10
Tcof1	Treacle protein	3	16
B4galt1	Beta-1,4-galactosyltransferase 1	2	12
Cdv3	Protein CDV3	2	7
Eif5b	Eukaryotic translation initiation factor 5B	2	15
Gstt3	Glutamine amidotransferase-like class 1 domain-containing protein 3, mitochondrial	2	5
Hexb	Beta-hexosaminidase subunit beta	2	2
Ighg1	Ig gamma-1 chain C region, membrane-bound form	2	4
Mmp11	Stromelysin-3	2	9
Neltfa	Negative elongation factor A	2	8
Park7	Parkinson disease protein 7 homolog	2	7
Parp1	Poly [ADP-ribose] polymerase 1	2	9
Pfdn6	Prefoldin subunit 6	2	7
Pnfa	PHD finger-like domain-containing protein 5A	2	10
Rps15	Small ribosomal subunit protein uS19	2	17
Rps27a	Ubiquitin-ribosomal protein eS31 fusion protein	2	11
Stk10	Serine/threonine-protein kinase 10	2	8
Tceg1	Transcription elongation regulator 1	2	4
Ubxn4	UBX domain-containing protein 4	2	11

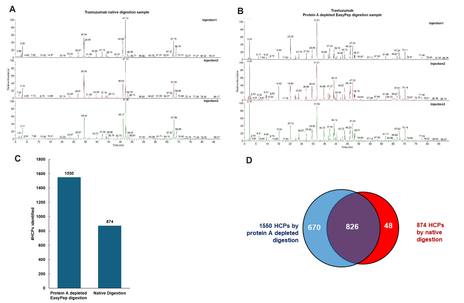
Comparison of different sample preparation methods for trastuzumab HCP

The large dynamic range of the biologic drugs compared to HCPs protein levels presents an analytical challenge for HCP detection and quantitation. Therefore, different sample preparation protocols are developed and optimized for HCP sample, such as the native digestion workflow we used, which provided satisfied HCP identification results with excellent reproducibility.

In this study, besides native digestion, we also evaluated the performance of a modified EasyPep sample preparation method that uses magnetic protein A beads based depletion for the detection of trastuzumab HCPs. The amount of HCP digested peptides was quantified using colorimetric peptide assay kit and 6.3 µg HCP digested peptides were loaded onto column.

Three replicate injections were acquired for both native digestion and Protein A depleted EasyPep digestion trastuzumab samples, using the same instrument settings as NISTmAb digested under native conditions. The BPC profiles were showed in Figure 4A and B. More chromatography peaks were separated and detected in Protein A depleted EasyPep digestion sample. This is explainable because this approach use magnetic protein A beads to remove the mAb, therefore enables denaturing digestion for HCPs, which leads to higher digestion efficiency for HCPs compares to native digestion. Figure 4C displayed the identified HCP numbers of native digestion and Protein A depleted EasyPep digestion. Three replicate injections were combined for database search using the Sequest HT and CHIMERYS intelligent search algorithm, and same protein/peptide level filters, as shown in Figure 2C. With these criteria, 1550 HCPs were identified using Protein A depleted EasyPep digestion and 874 HCPs for native digestion, 826 HCPs were identified in both samples, 670 HCPs were only identified in Protein A depleted EasyPep digestion sample and 48 were unique in native digestion sample (Figure 4D).

Figure 4. The HCP results of trastuzumab using native digestion and Protein A depleted EasyPep digestion. A, BPC profiles of trastuzumab sample using native digestion, three replicate injections. B, BPC profiles of trastuzumab sample using Protein A depleted EasyPep digestion, three replicate injections. C, HCP identified numbers using two different sample preparation protocols, database searching was performed with Sequest HT and CHIMERYS intelligent search algorithm, the filters applied for searching result were also listed here. D, HCP overlapping between two sample preparation protocols.



As displayed in Figure 4D, most of the HCPs identified in the native digestion sample were also found in the Protein A depleted digestion sample. Table 4 lists and compares the top 20 HCPs identified in both native digestion and Protein A depleted EasyPep digestion samples, sorted by descending #PSMs. From Table 4, it is evident that the high-abundance HCPs identified using both sample preparation protocols were highly similar, demonstrating that neither protocol discriminates against high-abundance HCPs. As shown in Figure 4C and 4D, the Protein A depleted EasyPep digestion identified significantly more HCPs compared to native digestion, proving that the Protein A depleted EasyPep digestion is more effective at digesting HCPs, particularly low-abundance HCPs. This results in the identification of a greater number of low-abundance HCPs. Although native digestion still identified 48 unique HCPs, indicating that using different sample preparation protocols and integrating the final results can provide a more comprehensive HCP identification list, the Protein A depleted EasyPep method clearly outperforms in terms of HCP identification, especially for low-abundance HCPs. Additionally, while native digestion is more time and cost-efficient compared to Protein A depleted EasyPep digestion, the superior performance of the Protein A depleted method in identifying low-abundance HCPs may influence researchers' decisions.

Table 4. Subset of top 20 HCPs identified in trastuzumab using both native digestion and Protein A depleted EasyPep digestion, respectively. Left, native digestion. Right, Protein A depleted EasyPep digestion. HCPs were listed by #PSMs, descending order.

Native Digestion						Protein A depleted EasyPep digestion					
No.	Gene Name	# Unique Peptides	# Peptides	# PSMs	Protein Description	No.	Gene Name	# Unique Peptides	# Peptides	# PSMs	Group Description
1	Thbs1	35	36	492	Thrombospondin 1	1	Thbs1	68	70	1015	Thrombospondin 1
2	Clu	24	24	428	Clusterin	2	Clu	29	29	588	Clusterin
3		33	35	406	78 kDa glucose-regulated protein	3	Hsp90b1	53	54	566	Endoplasmin
4	Hsp90b1	39	40	389	Endoplasmin	4	H671_4g13041	37	41	540	Pyruvate kinase
5	H671_4g13041	32	32	351	Pyruvate kinase	5		39	41	531	78 kDa glucose-regulated protein
6		33	34	302	Elongation factor 2	6		49	50	511	Elongation factor 2
7	LOC100754792	19	34	298	Heat shock protein HSP 90-beta	7	Hspg2	97	97	496	Heparan sulfate proteoglycan 2
8	I79_020107	37	37	290	Hypoxia up-regulated protein 1	8	LOC100754792	26	47	430	Heat shock protein HSP 90-beta
9	Actg1	5	14	275	Actin gamma 1	9	Fasn	80	82	412	Fatty acid synthase
10		20	34	261	Heat shock protein 90, alpha (cytosolic), class A member 1	10	H671_3g9525	28	28	373	Calreticulin
11	H671_4g13251	19	30	258	Heat shock cognate protein	11	H671_4g13251	22	36	372	Heat shock cognate protein
12	Fasn	51	51	256	Fatty acid synthase	12	I79_020107	45	45	364	Hypoxia up-regulated protein 1
13	H671_3g9525	23	23	251	Calreticulin	13		2	43	362	Heat shock protein 90, alpha (cytosolic), class A member 1
14		30	30	232	Protein disulfide-isomerase	14	LOC113830659	26	26	360	phosphopyruvate hydratase
15	H671_7g18204	45	45	228	RNA helicase	15	H671_7g18204	64	64	359	RNA helicase
16	LOC113830659	20	22	214	phosphopyruvate hydratase	16	H671_1g3330	5	22	334	Tubulin beta chain
17	H671_2g5970	33	40	209	Myosin-9	17	H671_2g5970	56	69	326	Myosin-9
18	LOC100773325	35	35	196	ATP-citrate synthase	18		38	38	318	Protein disulfide-isomerase
19		27	27	193	Protein disulfide-isomerase	19		6	20	313	Elongation factor 1-alpha
20	H671_1g3330	4	16	192	Tubulin beta chain	20	Pxdn	58	58	298	Peroxidasin

Conclusions

- Demonstrate the capability of a new Orbitrap Excedion Pro BioPharma mass spectrometer for HCP profiling. 163 HCPs of NISTmAb were identified using native digestion with excellent reproducibility. 0.007 ppm HCP Glutathione S-transferase P was identified with at least two unique peptides in all three injections, proved the excellent data quality for very low abundant protein of this new instrument platform.
- Both Protein A depleted EasyPep digestion and native digestion can provide high confident and reproducible HCP identification results. Protein A depleted EasyPep digestion gave 1550 HCPs while native digestion gave 874. The high abundant HCPs from two different sample preparations were very similar.
- All experiments were conducted under analytical flow UHPLC conditions, which are user-friendly and easy to maintain, while also providing excellent reproducibility and robustness.

References

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