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Characterization of Adeno-associated viral proteins and related proteoforms using top-down approach on a LC-Orbitrap Tribrid MS platform

Reiko Kiyonami[1], Rafael Melani[1], Roberto Gamez[1], Kenneth Thompson[2], Chao Yan Liu[2], Min Du[1], [1]Thermo Fisher Scientific, San Jose, CA, USA; [2]Thermo Fisher Scientific, Frederick, MD, USA

ABSTRACT

Purpose: Develop a top-down AAV viral protein mass analysis method for the phosphosite localization of VP2 and protein sequence confirmation of VP3 clip.

Methods: An AAV6 sample was analyzed with a targeted top-down approach on a Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system using multiple fragmentation techniques. The top-down data were processed using the Thermo Scientific™ BioPharma Finder™ 5.1 Software.

Results: The developed top-down method was able to localize two phosphorylation sites unambiguously by the multiple fragmentation techniques including EThcD, HCD and UVPD. The multiple fragmentation methods also allowed confident VP3 clip sequence confirmation with increased sequence coverage.

INTRODUCTION

AAV capsid viral proteins (VPs) are important constituents of AAV products and play an important role in immunogenicity and tissue tropism in gene therapy. In-depth characterization of viral proteins, including their truncated forms and PTMs, is critical for developing and manufacturing AAV products to ensure their safety, quality, and efficacy. In our previous work, we applied multiple LC-MS/MS approaches (Intact, top-down and peptide mapping) for AAV6 viral protein characterization on a Thermo Scientific™ Orbitrap Exploris™ 480 MS [1,2] and identified multiple potential phosphorylation sites with peptide mapping approach.[2] However, it was difficult to localize the accurate site of the phosphorylation with the peptide mapping approach only because of the high sequence overlaps of the VP1 and VP2. Orbitrap Ascend Tribrid MS enables multiple fragmentation techniques with higher ion transfer efficiency for the high *m/z* ions and provides an ideal platform for the top-down analysis of intact proteins. In this study, we performed targeted top-down analyses using EThcD, HCD and UVPD on the HPLC-Orbitrap Tribrid MS platform for the phosphosite localization of VP2 and protein sequence confirmation of VP3 clip. For the targeted precursor ion selection, an additional intact protein analysis was carried out. The results are reported here.

MATERIALS AND METHODS

Sample Preparation

An AAV6 sample expressed in HEK293 cells was used. The AAV6 sample was buffer exchanged and concentrated into 80% H_2O/20% acetonitrile containing 5 mM TCEP and 0.1% formic acid using 30K centrifugal filter (Amicon® Ultra, 0.5 mL). The estimated protein concentration of the AAV6 sample was 0.2 µg/µL after buffer exchange and concentration.

HPLC conditions

For all experiments, chromatographic separations were carried out using a C4 stationary phase column (1.0 x 100 mm, 300 Å, 1.7 µm) on the Vanquish Horizon UHPLC system. The solvent A was water with 0.1% difluoroacetic acid and the solvent B was acetonitrile with 0.1% difluoroacetic acid. The column temperature was set to 80 ºC. The flow rate was 50 µL/min. The gradient condition used is listed in Table 1. The Orbitrap Ascend Tribrid mass spectrometer equipped with the HMRn+, UVPD and ETD options was used for MS data collection. The ESI source settings were shown in the Table 2. The MS settings for the intact mass analysis are shown in Table 3. The MS settings for the top-down mass analysis are shown in Table 4.

Data Analysis

Thermo Scientific™ BioPharma Finder™ 5.1 software was used for both intact protein and top-down data analysis.

Table 1. HPLC gradient condition

Time	Flow (ml/min)	%B
0	0.05	20
1	0.05	32
16	0.05	36
20	0.05	80
21.5	0.05	80
22	0.05	20
30	0.05	20

Table 2. ESI settings

Sheath gas	25
Aux gas	5
Sweep gas	0
Spray voltage (+V)	3500
Capillary temp. (°C)	320
Vaporizer temp. (°C)	125

Table 3. MS settings for intact

Scan range (m/z)	800-5000
Application mode	Intact
Pressure mode	Low
Resolution	15000 at *m/z* 200
RF lens (%)	60
AGC target value	150
Max inject time (MS)	50
Microscans	10
Source fragmentation (V)	15

Table4. MS settings for top-down

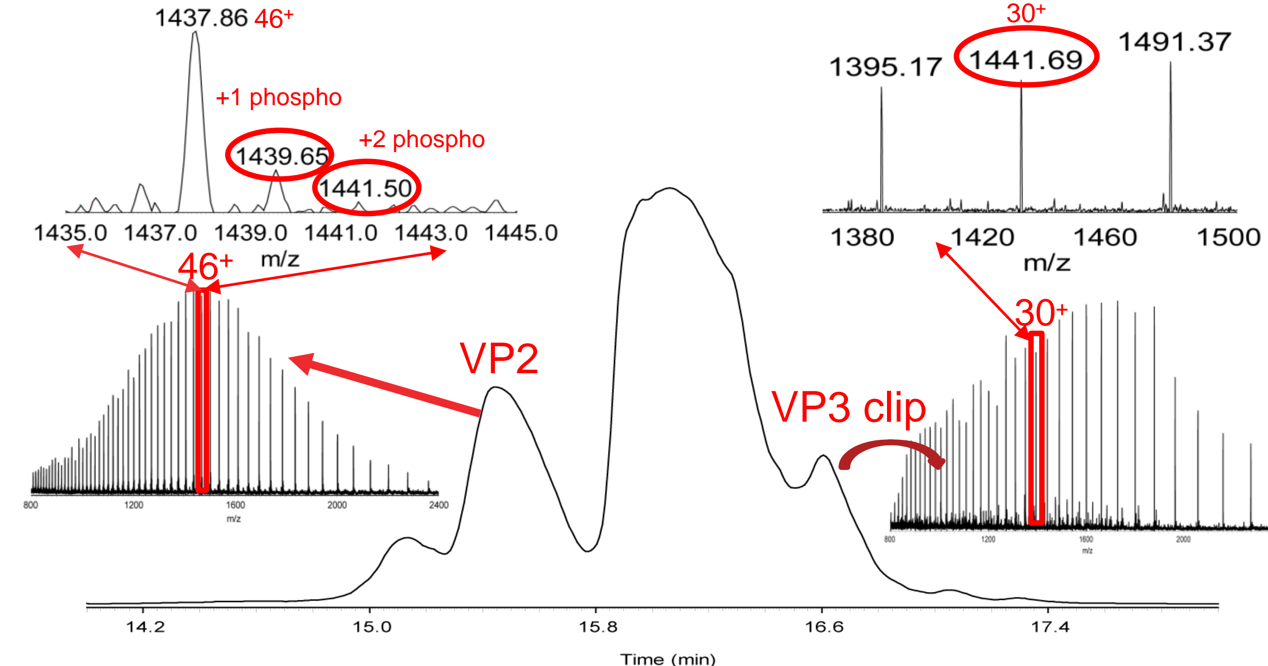
Full MS			
High mass range: 800-5000 mz; Intact low pressure mode; 7,500 resolution at m/z 200; 60 % RF lens; 150 % AGC target value; 5 micro scan; 15 v source fragmentation			
tMS2			
Targeted precursor ion	1441.5		
Isolation window	6		
Fragment type	EThcD	HCD	UVPD
Fragment parameters	reaction time:8 ms; Max. reagent inj.time:200 ms; Reagent target: 7E5; SA collision energy (%): 15	collision energy (%): 25	activation time: 25
Mass range	Normal	Normal	Normal
Scan range (m/z)	400-1400/1400-2000/400-2000	400-2000	400-2000
Application mode	Intact		
Pressure mode	Low		
Resolution (at m/z 200)	120,000		
RF lens (%)	60		
AGC target value	6,000		
Max inject time (MS)	2,000		
Microscans	3		

Results

Precursor ion selection for the targeted top-down experiments

An intact mass analysis was carried out first for the precursor ion selection. Figure 1 shows the TIC of the detected viral proteins and MS spectra of VP2 and VP3 clip. The enlarged *m/z* mass range for the charge 46 of VP2 clearly showed two phosphorylation sites (Figure 1, let insert). In order to generate the fragment ions directly linked to the two phospho-proteomeforms, *m/z* 1441.5 (the second phospho-proteoform) was selected as the precursor ion for all targeted top-down experiments. By using the isolation window of 6, the *m/z* 1439.65 (representing the first phospho-proteoform) was also co-selected. The selected precursor ion with isolation window of 6 also covers the charge 30 (*m/z* 1441.69) of VP3 clip and the acquired top-down data can be used not only for the phosphosite localization of VP2, but also for the protein sequence verification of VP3 clip.

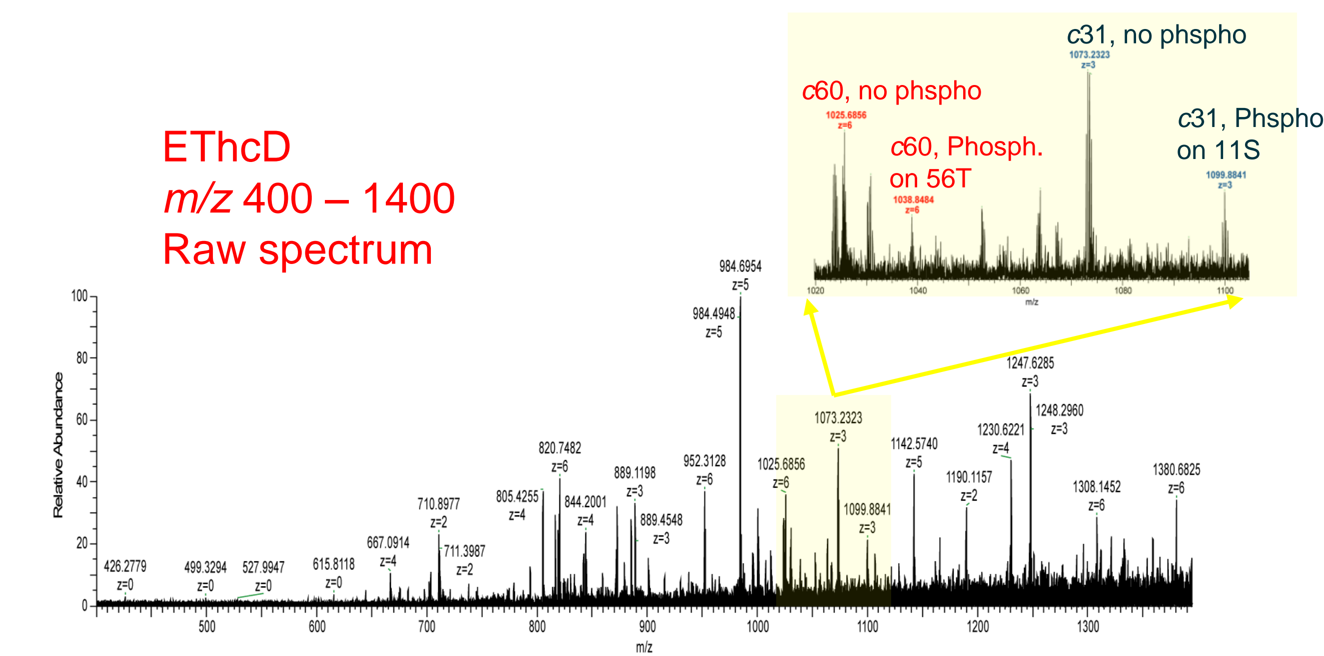
Figure 1. AAV6 viral protein intact mass analysis for precursor ion selection



Confident localization of two phosphorylation sites from VP2 using multiple fragmentation methods

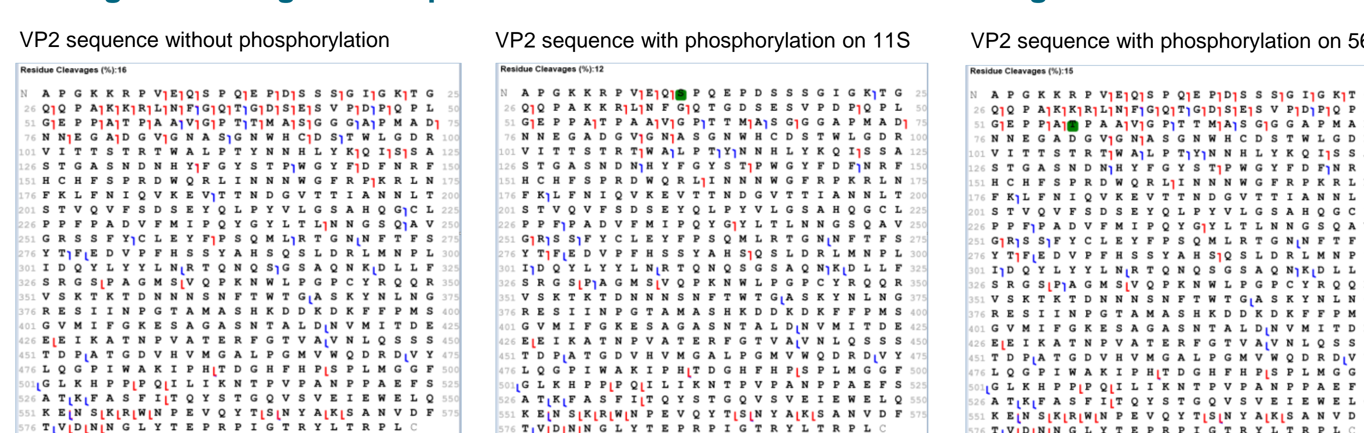
EthcD, HCD and UVPD were performed for the targeted top-down MS^2 experiments. A full MS scan was also performed in the same LC-MS run to ensure only the MS^2 data which are relevant to the VP2 or VP3 clip were used for data processing. Aimed to get more precursor ions into the c-Trap, high AGC value and up to 2000 ms ion injection time were used in all experiments. In order to get more fragments ions that are directly relevant to the phospho-proteome of VP2, we collected EThcD data using three different mass ranges. Figure 2 shows the fragment ions of EThcD collected with a mass range of *m/z* 400-1400. The *c*-type fragment pairs with and without phosphorylation in the specific amino acid locations were identified clearly (Figure 2, insert).

Figure 2. Fragment ions observed from EThcD with mass range of m/z400 - 1400



A total of five top-down raw files (Three EThcD, one HCD and one UVPD) were processed using the top-down workflow of Biopharma Finder 5.1 software. The fragment ion data across the eluted VP2 peak was averaged per raw file and the averaged spectra were searched against three VP2 proteoforms (VP2 proteoform without phosphorylation, VP2 phospho-proteoform with phosphorylation on the 11S and VP2 phospho-proteoform with phosphorylation on the 56T) using 15 ppm mass tolerance for the sequence coverage and phosphorylation localization confirmation. Figure 3 shows all three VP2 proteoforms were identified unambiguously with specific fragment ions using one of the EThcD (*m/z* 400 - 1400) data. The HCD and UVPD data search results provided complementary fragment ions for supporting the EThcD search results with increased sequence coverage. 26% sequence coverage was observed for the VP2 phospho-proteoform (11S) using UVPD (Figure 4). Overall, 50%, 45% and 47% sequence coverage were observed with the combined EThcD, HCD, UVPD data for the three VP2 proteoforms. Figure 5 shows the combined fragment map of the VP2 phospho-proteoform (56T) with the 47% sequence coverage.

Figure 3. Fragment maps observed from EThcD with mass range of m/z 400 - 1400



REFERENCES

1. Thermo Scientific application note # 000253. Reiko Kiyonami, et al., Highly sensitive intact mass analysis of AAV capsid proteins using a UHPLC-FLD-HRAM MS platform.
2. Thermo Scientific application note # 000242. Reiko Kiyonami, et al., Adeno-associated virus capsid protein characterization and host cell protein profiling using micro-flow UHPLC-Orbitrap MS.

Figure 4. UVPD search result for VP2 phospho-proteoform (11S)

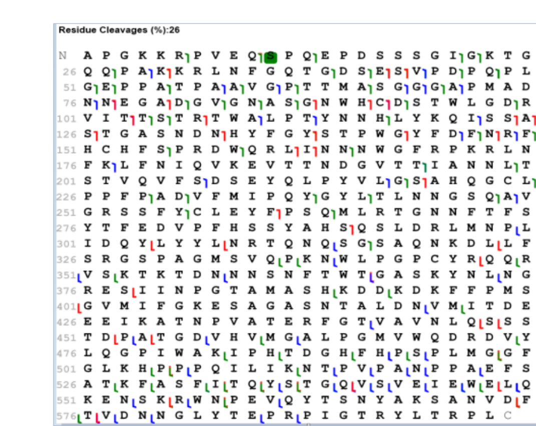
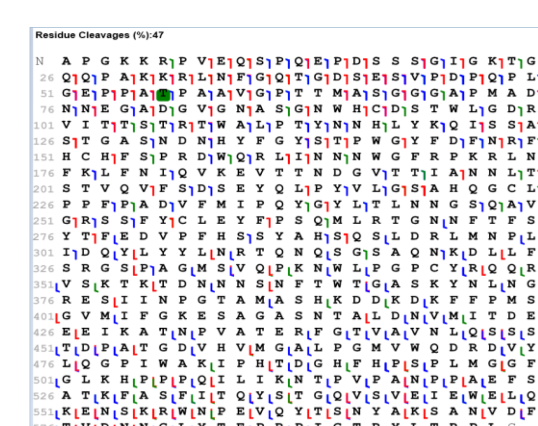


Figure 5. Combined search result for VP2 phospho-proteoform (56T)



Increased sequence coverage for the truncated VP3 proteoform using multiple fragmentation methods

The fragment ion data across the eluted VP3 clip peak was averaged per raw file and searched against the proposed Vp3 clip sequence[1] using 15 ppm mass tolerance. Multiple fragmentation methods provided significant numbers of unique fragment ions for the confident confirmation of the VP3 clip sequence (Figure 6). The sequence coverage observed using the combined fragmentation fragment ions was 50% (Figure 7).

Figure 6. Venn diagram of fragment ions which matched the VP3 clip sequence

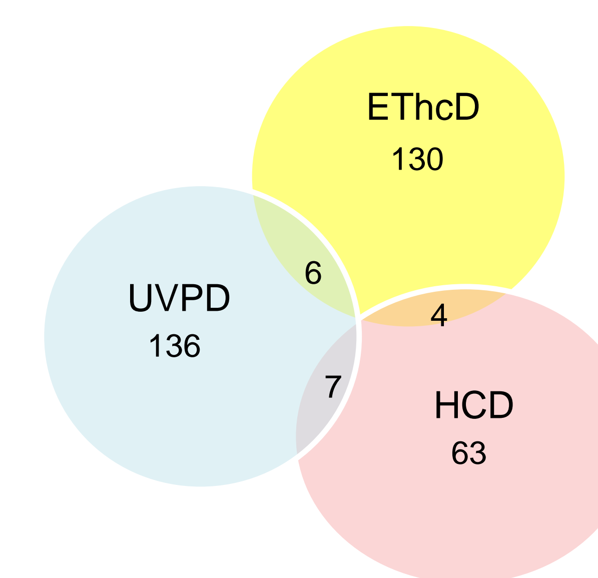
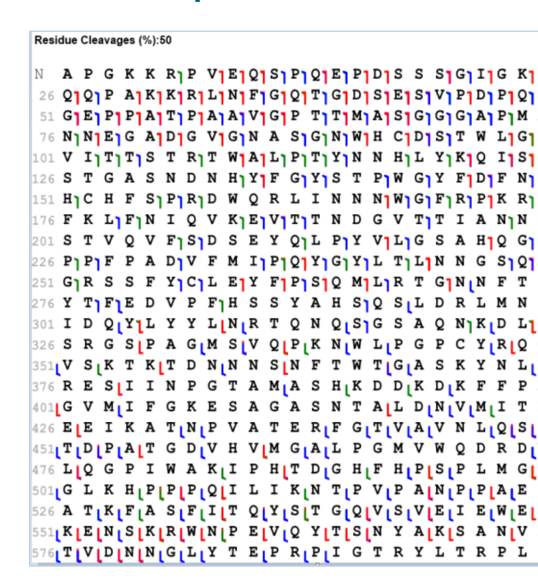


Figure 7. Combined search result for VP3 clip



CONCLUSIONS

- A targeted top-down method that uses optimized mass spectrometer settings and multiple fragmentation techniques was developed to address the challenges of AAV viral protein PTM characterization and truncated AAV protein sequence verification.
- The developed method was applied to direct fragmentation of low abundant VP2 phospho-proteofroms and VP3 proteoform with C-terminal truncation (VP3 clip).
- Two phosphorylation sites were verified unambiguously from VP2.
- The proposed VP3 clip sequence was confidently confirmed.
- The multiple fragmentation methods provided complementary fragment ions and enabled increased sequence coverage.

TRADEMARKS/LICENSING

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