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Characterization of Adeno-Associated Virus (AAV) Capsid Proteins with Liquid Chromatography Mass Spectrometry (LC-MS) Based Peptide Mapping and Post Translational Modification (PTMs) Analysis

Kate Xia[1], Stephen Kurzyniec[1], Evelyn Wang[1]
(1) Shimadzu Scientific Instruments, Columbia, MD 21046 U.S.A.

1. Introduction

Over the last three decades, adeno-associated viruses (AAVs) have emerged as widely utilized vectors in gene therapy due to their high transduction rates, wide tissue specificity, low immunogenicity, and minimal genotoxicity. The AAV capsid consists of three highly homologous viral proteins (VPs) known as VP1, VP2, and VP3. The comprehensive characterization of these VPs is crucial due to their pivotal role in viral infectivity and vector potency.

We used a bottom-up approach and LCMS-9050 Q-TOF to characterize AAV capsid proteins and PTMs with high sensitivity and accuracy.

2. Methods

In this study, AAV8 empty capsid reference material was purchased from Virovek (Houston, TX, USA) and used without further purification. The AAV sample was denatured in 6 M urea and 1 mM DTT at 80 ℃ for 30 minutes, followed by alkylation with 15 mM iodoacetamide for 30 minutes at room temperature in the dark. The reduced and alkylated samples were cooled to room temperature and diluted with three equivalent volumes of buffer (50 mM Tris-HCl and 1 mM CaCl2 [pH 7.5]). The samples were then digested overnight at 37 ℃ with 0.4 µg trypsin or chymotrypsin. The digestion was terminated by the addition of formic acid to 10% of final concentration and the sample was directly injected to the LCMS-9050 for analysis. The separation was achieved on a Shim-pack Velox C18 column (150mm x 2.1mm, 2.7µm).

3. Results

Digestion of AAV capsid proteins. Trypsin and chymotrypsin were employed to digest AAV capsid proteins. AAV8's sequence contains multiple regions rich in lysine and arginine residues, making it challenging to obtain the complete sequence using only trypsin. Therefore, chymotrypsin was utilized to improve sequence coverage. Both enzymes were able to digest AAV8 efficiently, resulting in populated chromatograms with a larger number of peptide peaks (Figure 1).

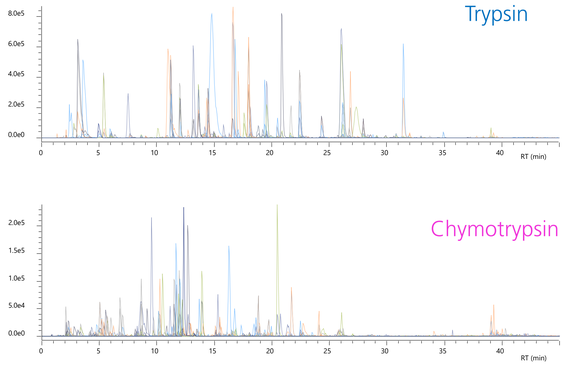

Figure 1: Extracted ion chromatogram (XIC) of peptide fragments from enzyme digested AAV8. Top: trypsin. Bottom: chymotrypsin.

Sequence coverage. In the PTM workflow of Protein Metrics software, all matched peptides were set to have a mass error of less than 6 ppm and at least one confirmatory MS/MS spectrum. The allowance for missed cleavages was set to 3. Carbamidomethyl was designated as a fixed modification, while variable modifications included oxidation, deamidation, pyro-Glu formation, ammonia loss, acetylation and phosphorylation. Most identified peptides exhibited excellent mass accuracy, with errors less than 1 ppm.

Table 1: Summary of sequence coverage

	Trypsin	Chymotrypsin	Total Coverage
VP1	93.90%	77.10%	96.07%
VP2	95.01%	81.53%	97.50%
VP3	97.19%	93.26%	100%

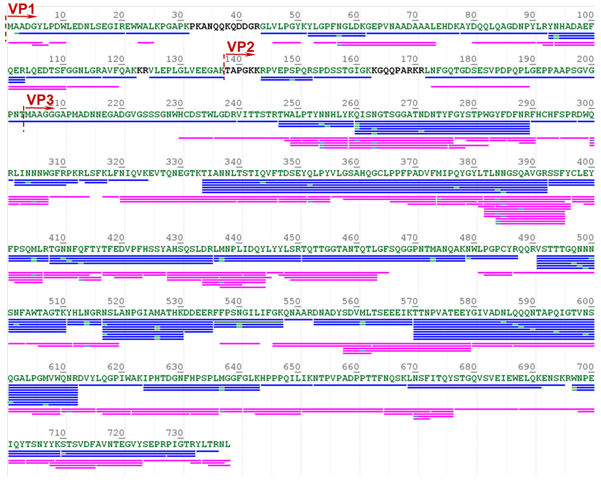

Figure 2: Sequence coverage map of AAV8 capsid proteins (VP1, VP2, and VP3) by LCMS-9050 using different enzymatic digests. Blue: trypsin. Magenta: chymotrypsin.

Post translational modifications (PTMs). Common PTMs such as deamidation, oxidation, acetylation, phosphorylation and pyroglutamate formation were found by Protein Metrics software. Figures 3 and 4 illustrate examples of these modifications. In Figure 3, the peptide TWALPTYNNHLYK (VP1 247–259), present in VP1, VP2, and VP3 (see Figure 2), is shown in both its native and deamidated forms. A +0.98 Da mass shift in the y6–y9 fragment ions (highlighted in green) confirms deamidation at an asparagine residue.

Similarly, Figure 4 shows the MS/MS spectra of the native and the oxidized peptides of SSFYCLEYFPSQMLR (VP1 393 – 407) , where the y6-y9 fragment ions (green boxes) all show the signature mass shift of 15.99 Da, indicating the presence of oxidation.

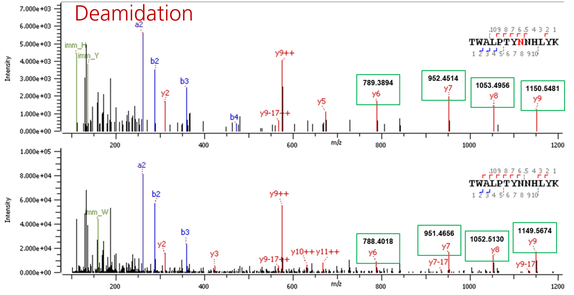

Figure 3: Asparagine deamidation analysis. Top: deamidation at Asn 254. Bottom: native peptide.

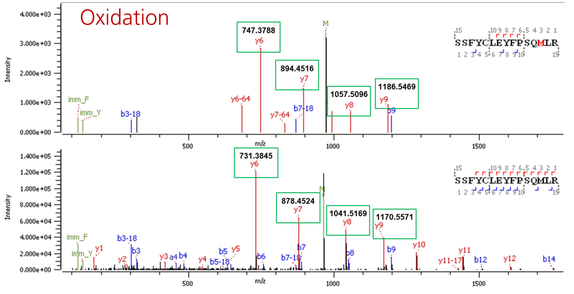

Figure 4: Methionine oxidation analysis. Top: oxidation at Met 405. Bottom: native peptide.

4. Conclusion

AAVs show strong potential as gene therapy vectors, making capsid protein CQA characterization essential for drug approval. This application presents a complete workflow—from sample prep to LCMS analysis—using trypsin and chymotrypsin for near-complete sequence coverage. High-quality MS/MS spectra enable confident peptide ID and PTM localization. The Shimadzu LCMS-9050 delivers reliable, accurate analysis of AAV capsid proteins.