Comprehensive characterization of an engineered Cas9 protein and its post-translational modifications (PTMs) by LC-MS/MS

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ABSTRACT

The CRISPR/Cas9 system has been increasingly used as a genome editing tool with applications that include disease treatment. The Cas9 endonuclease can cleave off-target sites, resulting in disruptions to the functionality and stability of normal genes. To overcome the frequent off-target cleavage, engineered Cas9 variants with amino acid mutations have been developed. PTMs that can modify protein structure and hydrophobicity also have an important impact on protein function and stability. Thus, a comprehensive characterization of engineered Cas9 that can confirm its amino acid sequence and PTMs is important to ensure its efficacy. Here, an LC-MS/MS approach was applied for the characterization of an engineered Cas9 protein and achieved >95% sequence coverage and confident identification of PTMs by electron activated dissociation (EAD).

INTRODUCTION

The CRISPR/Cas9 system has been increasingly used as a genome editing tool with applications that include disease treatment. The Cas9 endonuclease can cleave off-target sites when the single guide RNA (sgRNA) recognizes genome *loci* that are similar to the target DNA, resulting in disruptions to the functionality and stability of normal genes.[1] To overcome the frequent off-target cleavage by the wild-type Cas9 endonuclease, engineered Cas9 variants with amino acid mutations have been developed.[2] On the other hand, PTMs that can modify protein structure and hydrophobicity also have an important impact on protein function and stability. Thus, a comprehensive characterization of engineered Cas9 that can confirm the amino acid sequence and PTMs is important to ensure its efficacy.

A comprehensive characterization of an engineered Cas9 protein using Zeno trap-boosted EAD is presented here. EAD led to >95% sequence coverage of the Cas9 protein, excellent fragmentation of long peptides, differentiation between deamidation of isoAsp and Asp residues, and superior PTM confirmation by preserving side chain information. In addition, the highly sensitive collision-induced dissociation (CID) enabled confident identification of peptides with low-abundant mutations

MATERIALS AND METHODS

Sample preparation: The Cas9 protein was processed through a single-spot, solid-phase enhanced sample preparation strategy (SP3)[3]. Briefly, Cas9 protein was dissolved in 2% SDS buffer in 100mM ammonium bicarbonate (ABC) and heated at 60ºC for 5 min. Then, 2 µL each of hydrophobic and hydrophilic carboxylate-modified magnetic beads were added to the protein solution, followed by an addition of acetonitrile (ACN) so that ACN% was >70% by volume. The protein solution was mixed and incubated at room temperature for 18 min. A magnet was placed under the container to separate the magnetic beads from the supernatant. The supernatant containing SDS was carefully removed by pipetting. The beads were then rinsed twice using pure acetonitrile. The magnetic beads were resuspended with 100mM ABC buffer. Lys-C or Glu-C was added into the protein solution with an enzyme-to-protein ratio of 1:20. Enzymatic digestion was performed at 37ºC overnight.

The supernatant containing peptides was collected and acidified by formic acid (final pH = 3) after digestion.

LC-MS/MS analysis: The peptides were analyzed by an LC-MS system equipped with a Waters H-class HPLC for LC separation and a SCIEX ZenoTOF 7600 system for MS analysis. The peptides were separated with an ACQUITY CSH C18 column (2.1 × 150 mm, 1.7 µm, 130 Å, Waters). Gradient information can be found in reference 4. The flow rate was set to 0.2 mL/min. The column temperature was set to 60ºC. Mobile phase A was water, and mobile phase B was acetonitrile. Both contained 0.1% formic acid. LC-MS/MS data were acquired in SCIEX OS software using a ZenoTOF 7600 system in data-dependent acquisition (DDA) mode. MS parameters settings can be found in reference 4.

Data processing: CID and EAD DDA data were processed using the peptide mapping workflow templates in the Biologics Explorer software. The mass tolerances for MS and MS/MS were set to 10 ppm and 20 ppm, respectively. The instrument type was set to "CID (Glycopeptides)" and "EAD" for processing CID and EAD DDA data, respectively. Lys-C or Glu-C were chosen as enzymes for digestion with a maximum of 3 missed cleavages. The variable modifications included deamidation at Asn and Gln, acetylation at the N-terminus and Lys, phosphorylation at Ser, Thr, and Tyr, Ala-Arg and Arg-Ala substitutions and oxidation at Met. All other settings were set to the default.

RESULTS

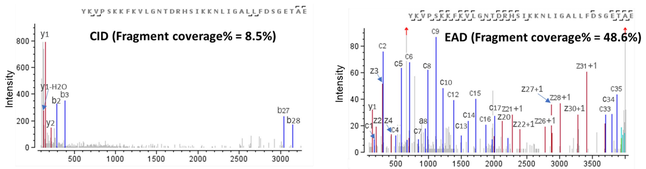

Figure 1. Comparison of a long peptide MS/MS spectra acquired using CID and EAD. The left panel shows a spectrum from CID fragmentation of the peptide YKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAE. The right panel shows a spectrum from EAD fragmentation of the same peptide. EAD resulted in significantly higher fragment coverage than CID for a long peptide with 36 amino acid residues.

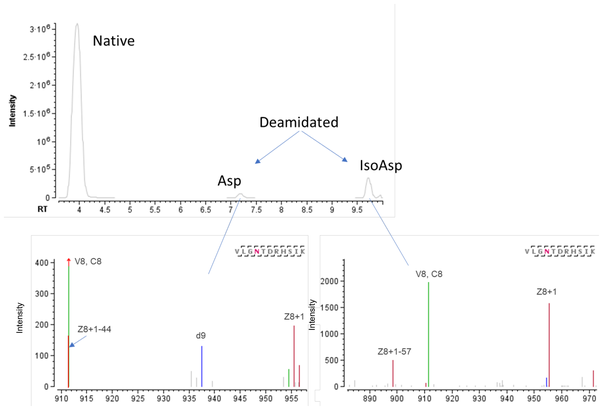

Figure 2. Differentiation of Asp and isoAsp by EAD. The top panel shows extracted ion chromatograms (XICs) of the native and deamidated peptide, VLGNTDRHSIK. The bottom panels show the MS/MS spectra of the peptide with Asp and isoAsp. The z8+1-44 ion highlighted in red in the bottom left panel indicates that the deamidated Asn is Asp. The z8+1-57 ion highlighted in red in the bottom right panel indicates the deamidated Asn is isoAsp.

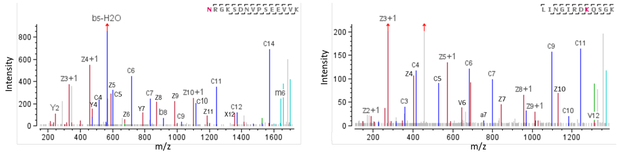

Figure 3. MS/MS spectra from EAD of peptides with acetylation. The left panel shows a representative spectrum of N-terminus acetylation. The right panel shows a representative spectrum of Lys acetylation.

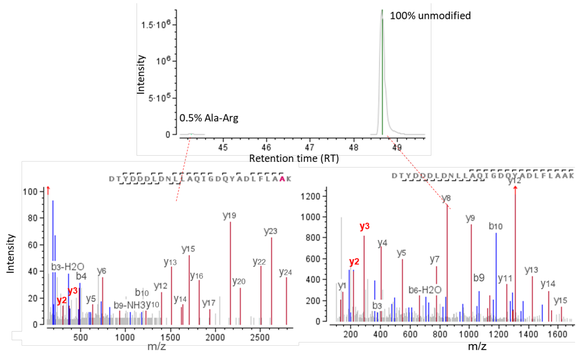

Figure 4. Identification of the Ala-Arg modification on peptide DTYDDDLDNLLAQIGDQYADLFLAAK. Top panel) XICs of the modified and unmodified peptides. When the abundance of the unmodified peptide is scaled to 1, the modified peptide only has 0.5% relative abundance. Bottom panels) Spectra from CID of the modified peptide (left) and unmodified peptide (right). The Ala-Arg substitution is highlighted in red in the sequence in the left panel. The fragment ions that indicate the mass shift are highlighted in red in the spectra.

CONCLUSIONS

- Comprehensive characterization and >95% sequence coverage of an engineered Cas9 protein were achieved with the ZenoTOF 7600 system
- EAD analysis was advantageous for:
  - -Identifying long peptides
  - -Confidently identifying and localizing acetylation modifications on the N-terminus and Lys residues
  - -Confidently differentiating between Asp and isoAsp residues
- Zeno trap activation with CID analysis allowed sensitive and confident identification of low-abundant peptides.
- The Biologics Explorer software showcased the identification and localization of PTMs and elucidated the relative quantity of the peptides in an XIC profile. Artificial PTMs during peptide sample preparation can also be detected.

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