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

Featuring the ZenoTOF 7600 system and Biologics Explorer software from SCIEX

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This technical note presents a comprehensive characterization of an engineered Cas9 protein using Zeno trap-boosted electron activated dissociation (EAD). 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 (Figure 1).

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.

In this technical note, an LC-MS/MS approach was applied for a comprehensive characterization of an engineered Cas9 protein that achieved >95% sequence coverage and confident identification of PTMs.

Key features of comprehensive Cas9 characterization

- The highly sensitive LC-MS/MS approach using the ZenoTOF 7600 system leads to >95% sequence coverage of the engineered Cas9 protein, which is essential to identify amino acid mutations
- EAD permits the confident identification of long peptides and the differentiation between deamidation and iso-deamidation
- EAD enables sites of N-terminus and Lys acetylation to be identified and localized with confidence
- The highly sensitive CID allows confident identification of peptides with low-abundant mutations
- The Biologics Explorer software provides a complete solution to identify and relatively quantify peptides

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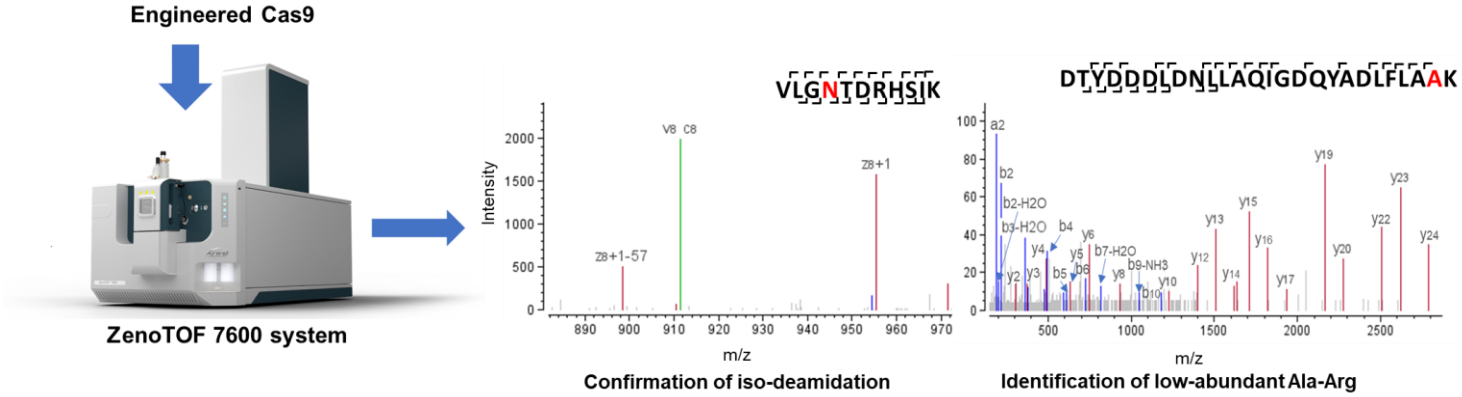

Figure 1. Peptide mapping results for the engineered Cas9 using LC-MS/MS. Nearly complete sequence coverage was achieved when results from the Glu-C and Lys-C digestions were combined. EAD allowed differentiation of Asp and isoAsp residues. Highly sensitive CID led to the confident identification of peptides with a low-abundant Ala-Arg substitution.

Methods

Chemicals and materials: Sodium dodecyl sulfate (SDS, P/N: L4390), acetonitrile (P/N: 34851-4L) and ammonium bicarbonate (ABC, P/N: A6141) were purchased from Sigma-Aldrich. Formic acid (P/N: A117-50) was purchased from Fisher Scientific. Lys-C (P/N: VA1170) and Glu-C (P/N: V1651) were purchased from Promega. Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (P/N: 45152105050250 and 65152105050250) were purchased from Cytiva.

Sample preparation: The Cas9 protein was processed through a single-spot, solid-phase enhanced sample preparation strategy (SP3). SP3 allows protein denaturing with detergents such as SDS and organic solvents. SP3 also allows MS-incompatible contaminants, such as SDS, to be efficiently removed prior to digestion.[3] Briefly, 10 µL of Cas9 protein (12 mg/mL) was dissolved in and diluted 10-fold by 2% SDS buffer in 100mM ABC. The protein was denatured by heating at 60°C for 5 min. Then, 2 µL of the hydrophobic and hydrophilic carboxylate-modified magnetic beads were added to the protein solution. Pure acetonitrile was added to the protein solution to yield a solution that was >70% acetonitrile by volume. The protein solution was mixed and incubated at room temperature for 18 min. Under high organic phase conditions in SP3, proteins have strong Hydrophilic interaction liquid chromatography (HILIC) interactions with magnetic beads when SDS is in the supernatant[3]. 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 200 µL of pure acetonitrile. The magnetic beads were resuspended with 90 µL of 100mM ABC buffer. The final Cas9 protein concentration was approximately 1-2 µg/µL. 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 37°C overnight.

After digestion, the solution containing beads was thoroughly mixed. The supernatant and magnetic beads were then separated by placing a magnet under the container. The supernatant containing peptides was then collected and acidified by formic acid (final pH = 3).

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) using the gradient shown in Table 1. The flow rate was set to 0.2 mL/min. The column temperature was set to 60°C. Mobile phase A was water and mobile phase B was acetonitrile. Both contained 0.1% formic acid.

Table 1. LC gradient.

Time (min)	A (%)	B (%)
Initial	*98*	*2*
2	*98*	*2*
60	*50*	*50*
62	*20*	*80*
72	*20*	*80*
74	*98*	*2*
80	*98*	*2*

LC-MS/MS data were acquired in SCIEX OS software using a ZenoTOF 7600 system in data-dependent acquisition (DDA) mode. The DDA parameters used in these experiments are shown in Tables 2 and 3.

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 M. All other settings were set to the default.

Table 2. TOF MS parameters.

Parameter	Value
Spray voltage	*5,500 V*
Ion source gas 1	*40 psi*
Ion source gas 2	*40 psi*
Curtain gas	*35 psi*
TOF start mass	*200 m/z*
TOF stop mass	*2,000 m/z*
Accumulation time	*0.1 s*
Source temperature	*375°C*
Declustering potential	*80 V*
Collision energy	*10 V*
Time bins to sum	*8*

Table 3. TOF MS/MS parameters.

Parameter	EAD	CID
Accumulation time	*0.1 s*	*0.06 s*
TOF start mass	*100 m/z*	
TOF stop mass	*2000 m/z*	
Electron beam current	*5,500 nA*	*NA*
Zeno threshold	*100,000 cps*	
Fragmentation	*0 eV KE*	*Dynamic CE*
CE	*12 V*	*NA*
Maximum candidate ions	*10*	
Cycle time	*1.176*	*0.77*
Time bins to sum	*10*	
EAD RF	*100 Da*	*NA*
EAD reaction time	*10 ms*	*NA*

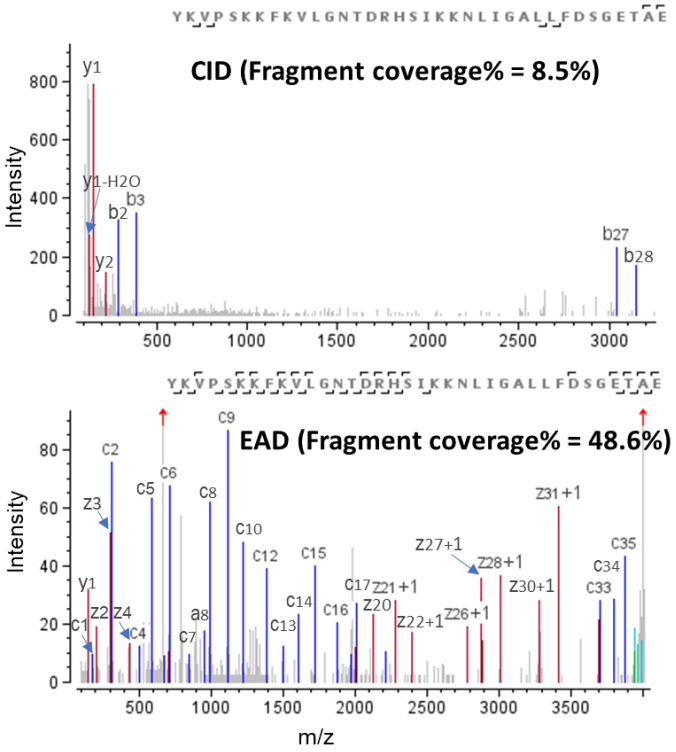

Figure 2. Comparison of long peptide MS/MS spectra acquired using CID and EAD. The top panel shows a spectrum from CID fragmentation. The bottom panel shows a spectrum from EAD fragmentation. EAD resulted in significantly higher fragment coverage than CID for a long peptide with 36 amino acid residues.

Cas9 amino acid sequence confirmation

Since the sequence of the engineered Cas9 contains only 2 cysteine residues, reduction and alkylation were not performed on the protein during sample preparation. The cysteine-containing peptides were identified. The Cas9 protein was digested overnight at 37°C using Glu-C or Lys-C with an enzyme-to-protein ratio of 1:20. The digested peptides were analyzed by LC-MS/MS on the ZenoTOF 7600 system using either EAD or CID for fragmentation. When EAD was used for fragmentation, 95% sequence coverage of Cas9 was achieved when data from Glu-C and Lys-C digestion were combined. Using CID for fragmentation, 97% sequence coverage of Cas9 was achieved when data from Glu-C and Lys-C digestion were combined. The nearly complete sequence coverage achieved by both CID and EAD highlighted the sensitivity of the ZenoTOF 7600 system and is critical for localization of any mutation in engineered Cas9 protein.

It was reported that electron activated dissociation with a radical dissociation mechanism can identify longer peptides and achieve higher sequence coverage.[4] These advantages are also observed during peptide mapping analysis of Cas9. Comparing the spectra obtained from commonly identified long peptides (Figure 2), EAD clearly showed a more evenly distributed fragmentation pattern, more thorough fragmentation and significantly higher fragment coverage than CID, which led to higher confidence in peptide identification. This is crucial for confident confirmation of amino acid sequence, especially when missed cleavage is unavoidable during digestion in peptide mapping.

PTM identifications

During database searching of the Cas9 peptide mapping, common PTMs such as acetylation, deamidation and phosphorylation were set as variable modifications.

Deamidation of asparagine or glutamine residues might lead to decreased protein biological activity and should be monitored in comprehensive characterization.[5] However, it is challenging to differentiate Asp and isoAsp isomers using CID or elution order. EAD, on the other hand, can generate diagnostic ions of z-44 for Asp and z-57 for isoAsp, in addition to a parent fragment ion with 0.98 Da mass shift. The diagnostic ion can identify deamidations and differentiate between Asp and isoAsp. Figure 3 shows an example of the identification of a deamidation mutation (Asp and isoAsp) of peptide VLGNTDRHSIK using EAD. The diagnostic ion of z8+1-44 and z8+1-57 were clearly detected with good signal-to-noise. These results demonstrate the ability of EAD for

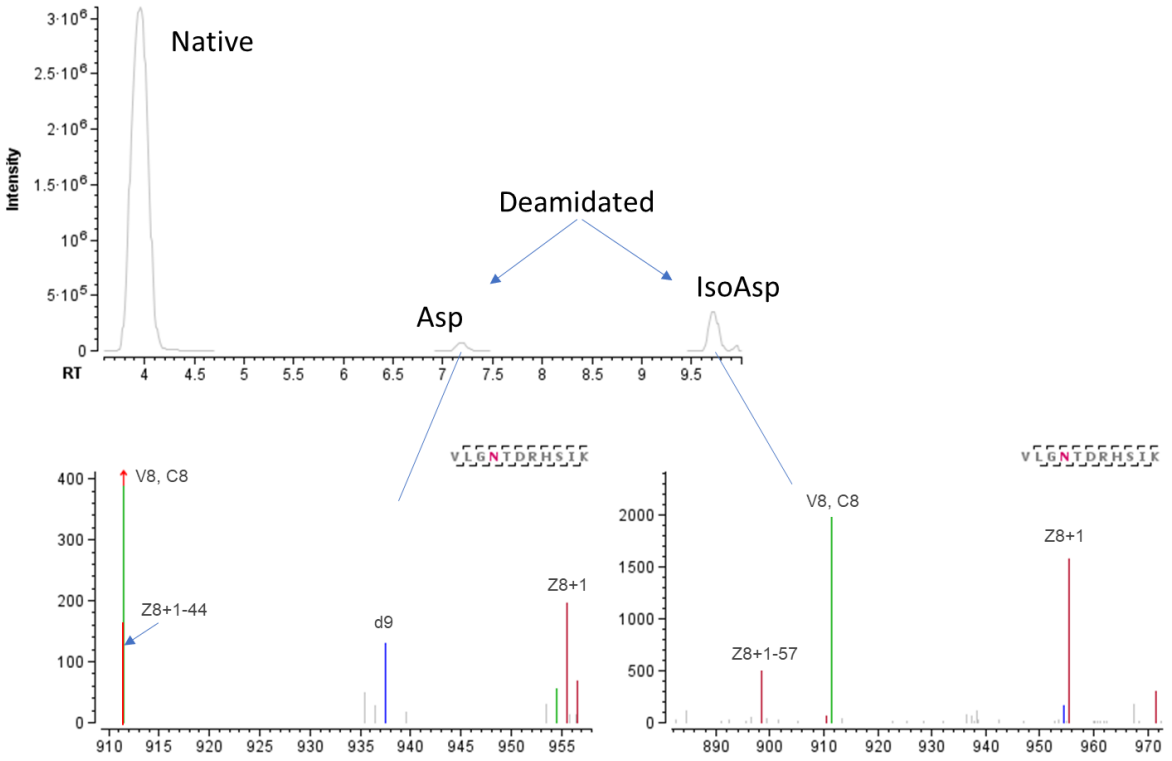

Figure 3. Differentiation of Asp and isoAsp by EAD. The top panel shows extracted ion chromatograms (XICs) of the native and deamidated peptide, VLGNTDRHSIK. The bottom panels show the MS/MS spectra of the peptide with Asp and isoAsp. The z8+1-44 ion highlighted in red in the bottom left panel indicates that the deamidated Asn is Asp, the z8+1-55 ion highlighted in red in the bottom right panel indicates the deamidated Asn is isoAsp.

accurate localization of deamidation sites and confident differentiation between deamidation isomers.

Acetylation can dramatically change the function of a protein through the alteration of protein hydrophobicity, solubility and surface properties, all of which might influence protein conformation and interactions with substrates.[6] Identification of multiple acetylations on Lys residues and on the N-terminus was achieved from EAD analysis of the engineered Cas9. Figure 4 shows representative spectra achieved from EAD of peptides with acetylation. Thorough fragmentation led to the accurate localization and identification of acetylation.

It was reported that Cas9 protein could go through Arg-Ala substitution.[7] Therefore, Arg-Ala and Ala-Arg substitutions were set as variable modifications in the database search. The Zeno trap of the ZenoTOF 7600 system enabled the ultrasensitive identification of peptides in low abundance. With the Zeno trap enabled, peptides with low-abundant PTMs could be identified with high confidence. For example, in the peptide, DTYDDDLDNLLAQIGDQYADLFLA**A**K, an Ala-Arg substitution was identified on the last Ala residue (bolded). The modified peptide had only 0.5% relative abundance compared

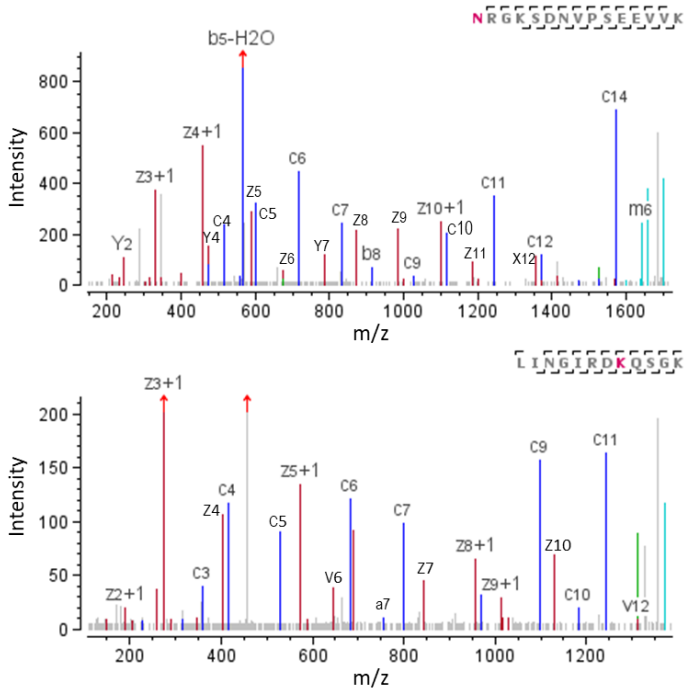

Figure 4. MS/MS spectra from EAD of peptides with acetylation. The top panel shows a representative spectrum of N-terminus acetylation. The bottom panel shows a representative spectrum of Lys acetylation.

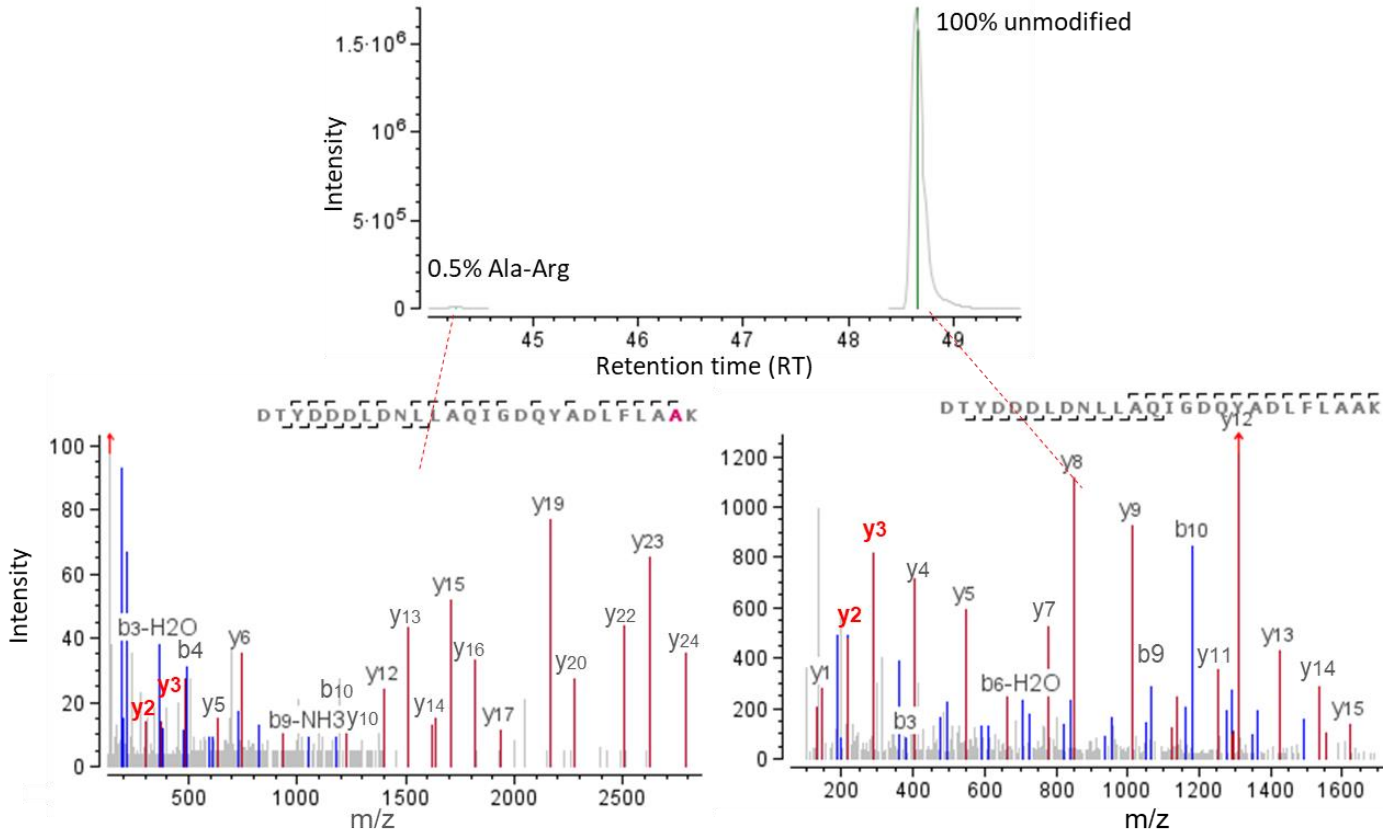

Figure 5. Identification of the Ala-Arg modification on peptide DTYDDDLDNLLAQIGDQYADLFLAAK. Top panel) XICs of the modified and unmodified peptides. When the abundance of unmodified peptide is scaled to 1, the modified peptide only has 0.5% relative abundance. Bottom panels) Spectra from CID of the modified 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.

to the unmodified version, according to the reported intensity from the Biologics Explorer software. The Biologics Explorer software showcased the extracted ion chromatogram (XIC) when both peptides were selected, providing a visualization of peptide quantity in chromatographic peak profile (Figure 5, top panel). The earlier elution of the modified peptide was consistent with the hydrophobicity change from Ala to Arg. With only 0.5% of the relative abundance of the modified peptide, the Zeno trap still allowed highly confident identification of the peptide and high fragment coverage with fragment ions obtained from CID (Figure 5, bottom panels).

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 and 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.

References

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