Investigation of Proteolytic Cleavage at Modified Residues

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OBJECTIVE:

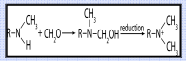
- **Investigate enzyme specificity on modified residues**
- **Study affect on proteolytic processing relative to size of chemical modifier on cleavage residue**

INTRODUCTION:

The identification and localization of posttranslational modification (PTM) sites on proteins can be key to understanding the mechanism of various cellular processes. Proteolytic enzymes are typically employed as part of the bottom up proteomic work flow for identifying post translationally modified peptides. Several criteria are commonly used to help verify and conclude if an identified peptide fragment is modified or not. Part of the characterization "dogma" is that modified residues typically cannot undergo proteolysis due to potential steric hindrance and reduced enzyme accessibility during proteolysis. Here we demonstrate data suggesting the widely accepted rule of thumb that enzymes cannot cleave at modified residues maybe not apply to all proteases.

In this study, we investigated the potential for proteolytic cleavage following protein level chemical labeling at enzyme specific residues. Trypsin and Lys-N were used to study Lysine modified Bovine Serum Albumin (BSA). Different size chemical modifiers were tested to evaluate impact on steric hindrance and enzyme accessibility. BSA was subjected to the following modifications: (1) Reductive Methylation (+28.01Da), (2) Propionylation (+56.03Da), and (3) Biotinylation (+226.08Da). Mass spectrometric data from an analysis of a Histone prep will also be discussed.

(1) Reductive Methylation



(2) Propionylation

(3) Biotinylation

MATERIALS AND METHODS:

❖ Bovine Serum Albumin (BSA) Analysis:

1. 0.2mg/mL BSA was reduced (Dithioreitol) and alkylated (Iodoacetamide) in 0.1M TEAB, pH 8.5 or 0.1M AMBIC, pH8.5
2. Samples were unlabeled (control) or subjected to 3 different labeling methods :
   a. Reductive Methylation[1] :
      i. Added 20ul of each reagent to sample: 12%CH_2O and 1.8M $NaBH_3CN$ (incubated overnight)
   b. Propionylation [2]:
      i. Added Propionic Reagent (1:3, propionic anhyride:isopropanol to sample)
      ii. Checked and adjusted to pH8.0 (incubated for 15minutes @ 37° C)
      iii. Dried down in speedvac to <5ul
      iv. Repeated steps i-iii
   c. Biotinylation:
      ➢ Added 10mM Sulfo-NHS-Biotin to sample (incubated on ice for 2 hours)
3. Excess labeling reagent was removed using a 7kDa Zeba spin column (Thermo Fisher)
   i. Samples were enzymatically digested in-solution with Lys-N or Trypsin, at a 1:100 enzyme: protein ratio at 37° C overnight.
4. All samples were subjected to ESI LC-MS/MS analysis on an LTQ-FT mass spectrometer (Thermo Fisher) interfaced with a nanoAcquity UPLC (Waters Corp).
5. Mass spectrometric results were searched using Mascot (Matrix Science) with the following variable modifications: Lysine and N-terminal dimethylation, Lysine Propionylation, Lysine Biotinylation, Methionine oxidation, and Cysteine carbamidomethylation. Downstream data processing was performed using GFY (under license from Harvard University).

Protein sample → Reduce/alkylate → Chemical Label → Quench Reaction → desalt → Enzymatic Digest → LC-MS/MS → Database Search (Mascot) → Data Process via GFY

❖ Histone Analysis:

1. Histones from murine primary T-cells were extracted using Histone Purification kit (Active Motif)
2. Samples were labeled and digested per Garcia lab protocol[2].
3. Samples were analyzed on an Orbitrap Velos Pro (Thermo Fisher) interfaced with a nanoAcquity UPLC (Waters Corp).

RESULTS AND DISCUSSION:

Reductive Methylation:

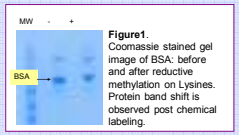


Figure1. Coomassie stained gel image of BSA: before and after reductive methylation on Lysines. Protein band shift is observed post chemical labeling.

Figure2. Protein Sequence Coverage of BSA unlabeled versus reductive methylated. a.) Control, no labeling b.) Reductive Methylation. The total number of peptides spectra matched (PSM) detected post digestion for the labeled BSA was much less than the unlabeled BSA.

Lys-N Digestion:

Peptide: A.K*EYEATLEECCA.K

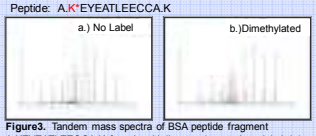


Figure3. Tandem mass spectra of BSA peptide fragment A.KEYEATLEECCA.K from Lys-N digest. a.) control, no label; b.) dimethylated Lysine

Trypsin Digestion:

Peptide: K.YICDNQDTISSK*.L

Figure5. Tandem mass spectra of BSA peptide fragment K.YICDNQDTISSK.L from Trypsin digest. a) control, no label; b) dimethylated Lysine

Peptide: F.K*DLGEEHF.K

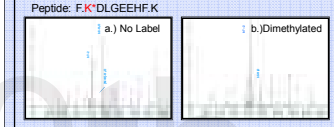


Figure4. Tandem mass spectra of BSA peptide fragment F.KDLGEEHF.K from Lys-N digest. a.) control, no label; b.) dimethylated Lysine

Peptide: K.LVNELTEFAK*.T

Figure6. Tandem mass spectra of BSA peptide fragment K.LVNELTEFAK.T from Trypsin digest. a) control, no label; b) dimethylated Lysine

Confirmation of Cleavage at Modified Lysine with AQUA Peptides:

Lys-N Digestion:

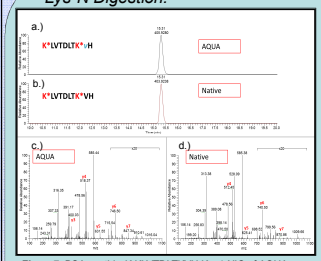


Figure7. BSA peptide K.KLVTDLTKVH.K a.) XIC of AQUA peptide, isotope label on *y*, b.) XIC of Native peptide, c.) MS/MS of AQUA Peptide, d.) MS/MS of Native Peptide. AQUA peptide was spiked into sample to confirm identification of the Lys-N digested peptide fragment.

Trypsin Digestion:

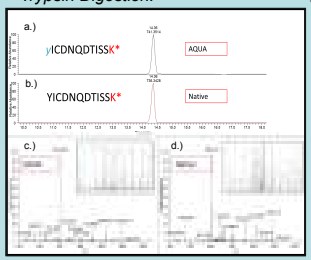


Figure8. BSA peptide K.YICDNQDTISSK.L a.) XIC of AQUA peptide, isotope label on *y*, b.) XIC of Native peptide, c.) MS/MS of AQUA Peptide, d.) MS/MS of Native Peptide. AQUA peptide was spiked into sample to confirm identification of the Trypsin digested peptide fragment. Inset in Figures 8c&d highlight b-fragment ion coverage.

More Miscleavages Observed When Proteolytic Residue is Modified

Table1. List of miscleaved peptides from Trypsin digestion, categorized by the chemical labeling. More miscleaved peptide fragments observed when proteins are chemically labeled at proteolytic residue.

	Trypsin Digestion of BSA			
	Control (No Label)	Reductive Methylation	Biotinylation	Propionylation
Total # of Miscleaved Peptides (PSM)	53	72	135	164
Total # of Peptides (PSM)	400	154	467	280
% of Miscleavage	13%	47%	29%	59%

Table2. Percentage of miscleaved peptides detected. The percentage of miscleaved peptide fragments for Trypsin digestion increases with the different chemical labeling methods compared to the control. Overall lower PSM count observed in chemically labeled samples than the unlabeled sample. This might be indicative of incomplete digestion due to the steric hindrance or reduced enzyme accessibility. Similar results observed for Lys-N digestion(data not shown).

RESULTS AND DISCUSSION:

Propionylation:

Lys-N Digestion:

Peptide: K.K*QTALVELLK*H.K

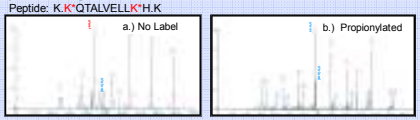


Figure9. Tandem mass spectra of BSA peptide fragment K.KQTALVELLKH.K from Lys-N digest. a.) control, no label; b.) Propionylated Lysine

Trypsin Digestion:

Peptide: R.RHPYFYAPELLYYANK*.Y

Figure10. Tandem mass spectra of BSA peptide fragment R.RHPYFYAPELLYYANK.Y from Trypsin digest. a.) control, no label; b.) Propionylated Lysine

Biotinylation:

Lys-N Digestion:

Peptide: E.K*SLHTLFGDELC.K

Figure11. Tandem mass spectra of BSA peptide fragment E.KSLHTLFGDELC.K from Lys-N digest. a.) control, no label; b.) Biotinylated Lysine

Trypsin Digestion:

Peptide: K.YICDNQDTISSKLK*.E

Figure12. Tandem mass spectra of BSA peptide fragment K.YICDNQDTISSKLK.E from Trypsin digest. a.) control, no label; b.) Biotinylated Lysine

Histone4: Tryptic Cleavage at Propionylated Lysine

Peptide: R.GKGGKGLGKGGAK*.R

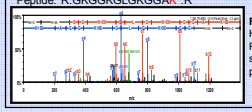


Figure13. Tandem mass spectra of Histone4 tryptic peptide fragment R.GKGGKGLGKGGAK.R detected in sample where Lysines are propionylated prior to enzymatic digestion.

CONCLUSION:

- Enzymes such as Lys-N and Trypsin can cleave at modified Lysines. In this study, proteolytic cleavage was observed even when Lysines were Dimethylated (Fig.3-8), Propionylated (Fig.9-10), or Biotinylated (Fig.11-12) at the protein level.
- It is not preferential for proteolytic cleavage to occur at modified residues, as seen in fewer PSMs (Fig2) in labeled versus unlabeled BSA.
- AQUA peptides KLVTDLTKVH and YICDNQDTISSK were used to confirm the identification of the Lys-N and Trypsin digested BSA peptide fragments with dimethylated Lysines (Fig.7-8).
- Proteolytically miscleaved peptide fragments observed in both Lys-N and Trypsin were more prevalent in the chemically labeled samples compared to the control. The frequency of miscleaved product correlates well with the size of the modification (Table1 & 2). This implicates incomplete digestion could be occurring due to steric hindrance or enzyme accessibility.
- A Histone4 peptide fragment where tryptic cleavage at propionylated Lysine was identified (Fig.13).

REFERENCES:

1. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nature Protocol. 2009;4(4):484-94.
2. B.A. Garcia, S. Mollah, B.M. Ueberheide, S.A. Busby, T.L. Muratore, J. Shabanowitz, D.F. Hunt. Chemical derivatization of histones for facilitated analysis by mass spectrometry. Nat. Protocols, 2 (2007), pp. 933–938.

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