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Characterization of carbamoylated lysine in a therapeutic recombinant protein using top-down electron fragmentation

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Introduction

The determination of processing artifacts and post-translational modifications (PTMs) in therapeutic recombinant proteins is important to ensure their biological activity and metabolism. Unwanted modifications on recombinant proteins can be generated during industrial production or storage, which requires quality control both at the molecular and functional levels. High molarity urea is frequently used in solubilization and the purification of recombinant proteins and it can lead to carbamoylation at protein/peptide N-terminal and lysine residues (Figure 1).

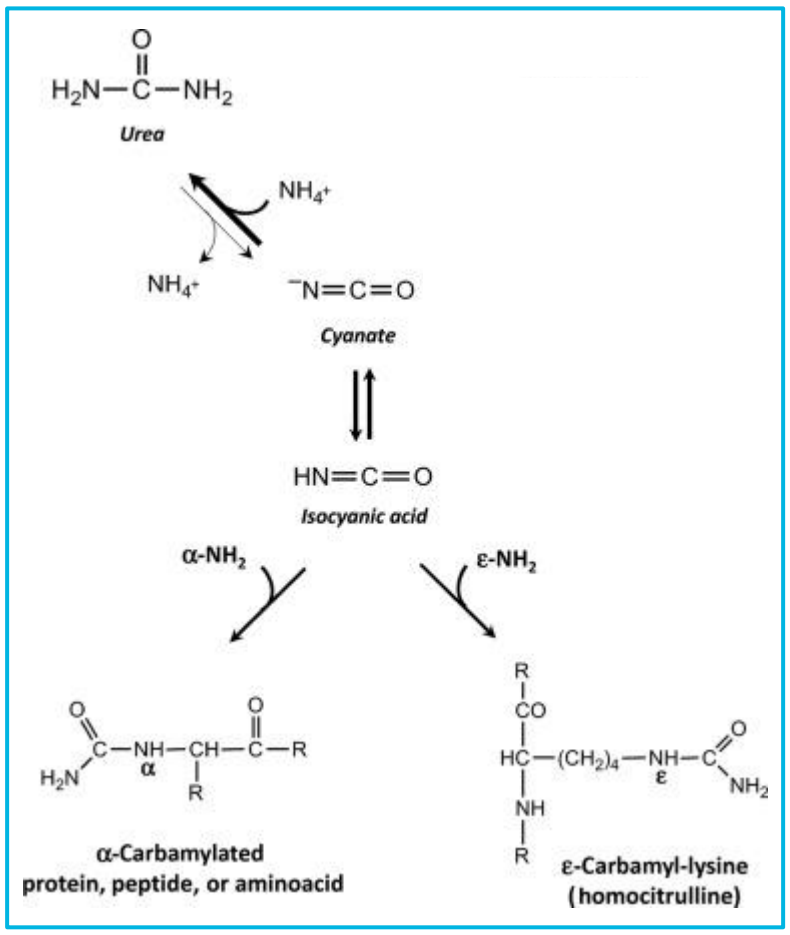

Figure 1. Protein carbamylation process[2]

Mass spectrometry (MS) is currently used to characterize the composition and structure of proteins. Electron Capture Dissociation (ECD) fragmentation produces clean mass spectra of intact proteins, while preserving labile PTMs in contrast to collision induced dissociation (CID), the most commonly used MS fragmentation method. Here, we used ECD implemented in high resolution MS instruments to analyze N-terminal and lysine carbamoylation in an experimental therapeutic recombinant protein requiring solubilization in urea.

Experimental

Protein production and purification

A prokaryotic protein containing 14 lysine residues (17 kDa) was recombinantly expressed in a prokaryotic system and purified using pH 8, 4-8M urea, 30-100 mM Tris-HCl buffer.

Sample preparation

For MS analysis, the protein was reduced with TCEP, precipitated in a phosphate buffer, centrifuged, and washed. The final precipitate was dissolved in a solution of 1% acetic acid.

MS analysis

The protein was introduced via direct infusion using an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer. The instrument was modified with a prototype ExD cell (e-Msion – A part of Agilent)) to enable ECD fragmentation (figure 2). ExDViewer and MassHunter Qualitative Analysis programs were used to analyze spectra.

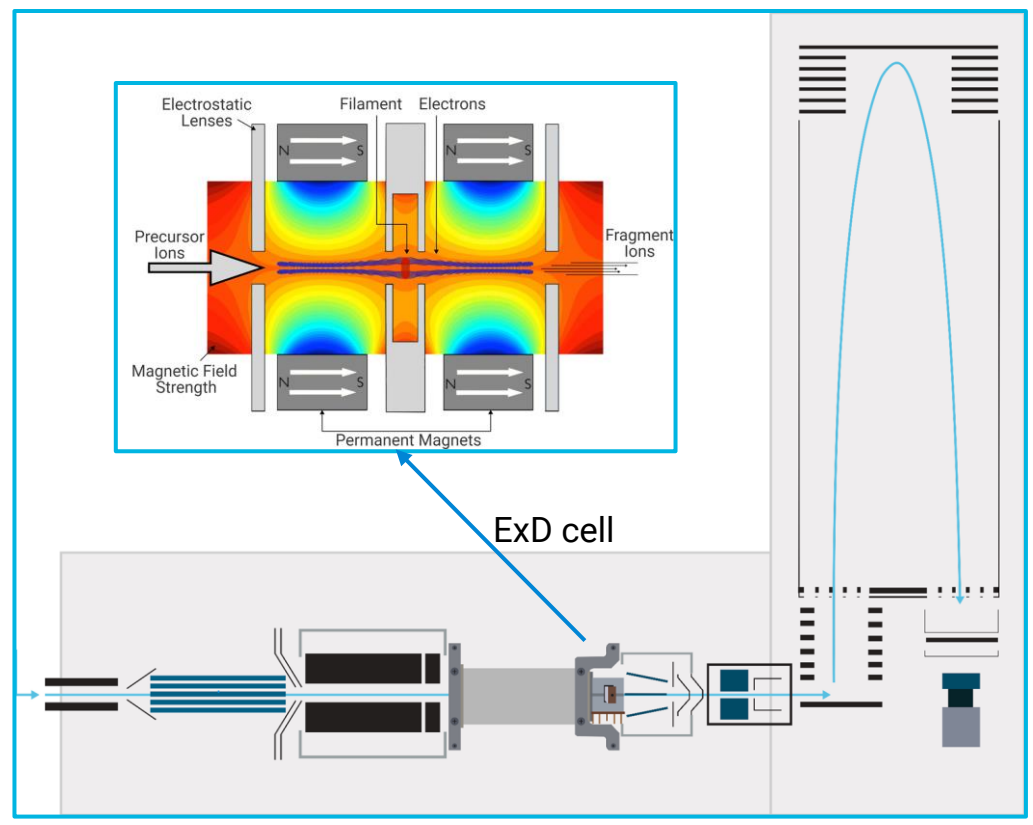

Figure 2. Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer modified with the ExD cell.

Results and Discussion

Intact mass reveals a mixture of modifications

The sequence of the recombinant protein contained multiple lysine residues, which theoretically were susceptible to undergo carbamoylation. As can be seen in figure 3, the MS1 profile showed pools of several molecular species with a mass difference of 43 Da, tentatively belonging to different PTMs associated with carbamoylation.

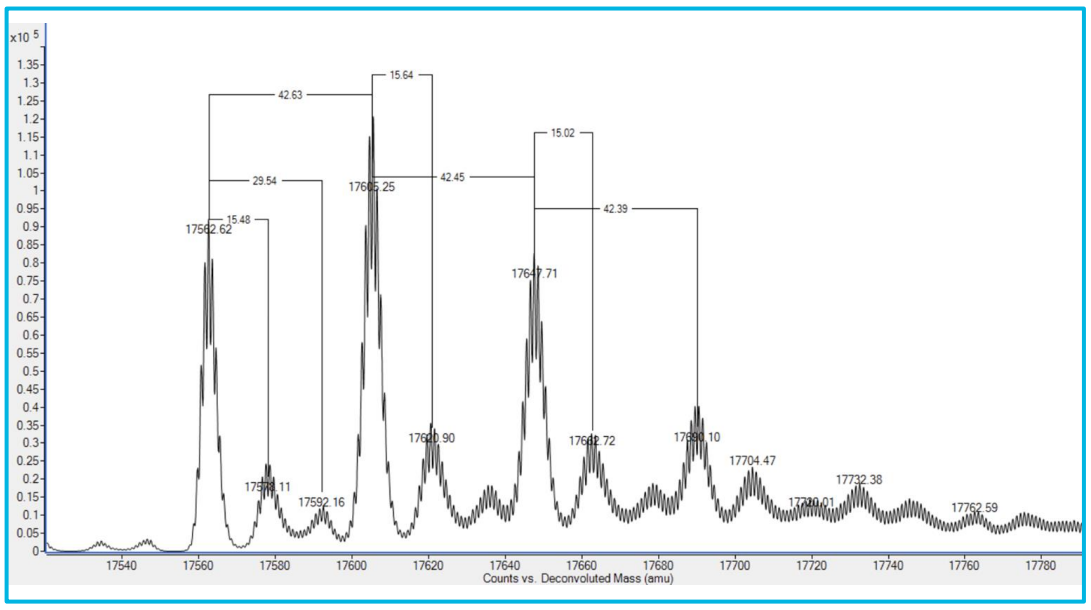

Figure 3. MS1 deconvoluted spectra using Agilent MassHunter software.

Top-down ECD fragmentation

For tandem MS, the whole isotope envelope was isolated and ECD was used to fragment the selected precursor to determine position of carbamoylation in proteins. Using the ExDViewer software, the tandem MS/MS spectra obtained allowed for the accurate identification of lysine and N-terminal carbamoylation (figure 4).

Confirmation of carbamoylation

To assess whether carbamoylation was due to exposure to 4-8M urea during the recombinant protein purification, the protein was purified in urea-free solutions. The resulting protein was not carbamoylated on lysine residues. Moreover, overnight exposure to 4-8M urea at room temperature was sufficient to induce extensive Lys carbamoylation corresponding to additions of +43 Da in MW (figure 5).

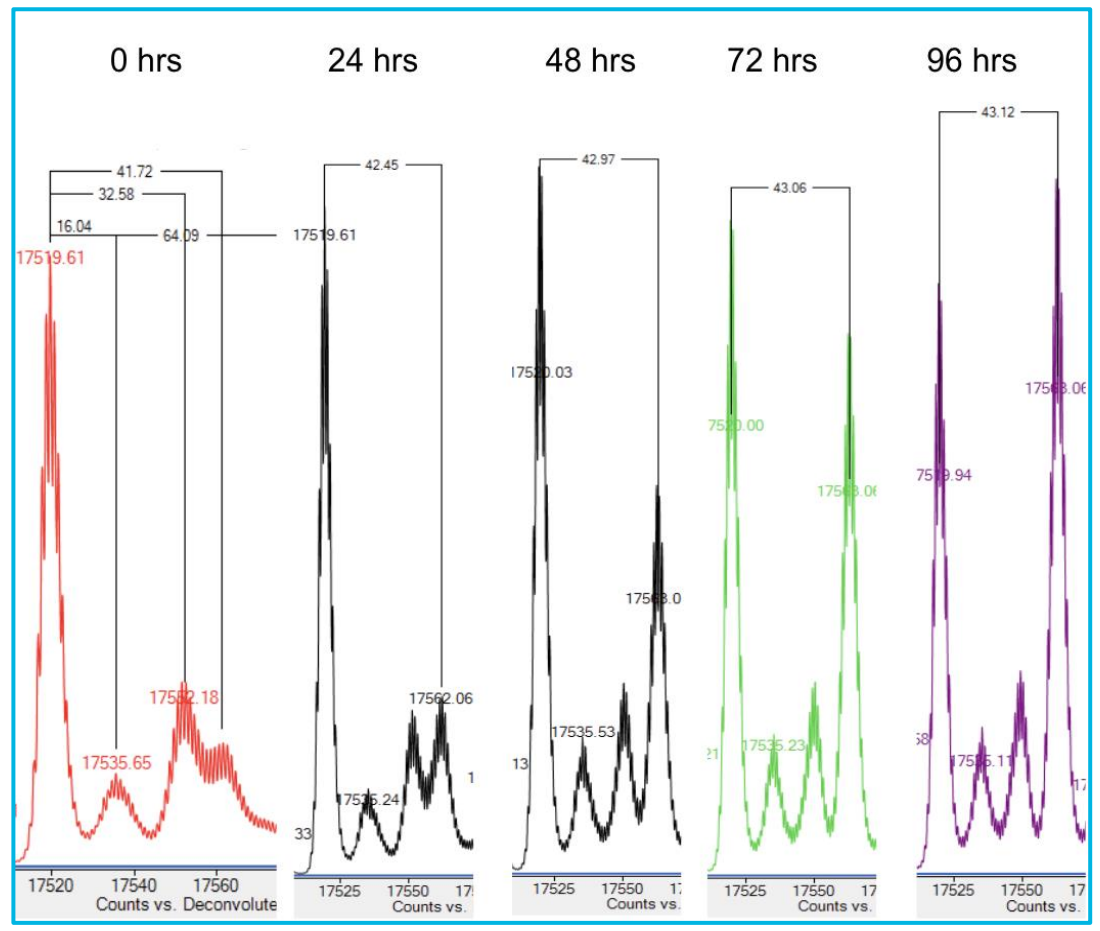

Figure 5. MS1 deconvoluted spectra of the protein incubated in urea buffer at different times. Agilent MassHunter software was used.

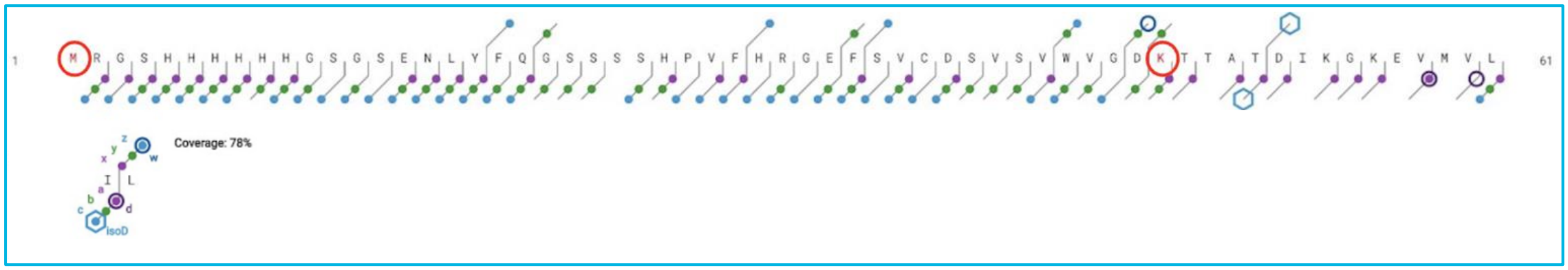

Figure 4. Characterization of selected sequence of carbamylated N-terminal and Lys (red circles) by MS/MS using ExDViewer software. Red circles represent carbamoylated residues.

Results and Discussion

ECD spectra analysis and assignment of peaks

One of the challenges faced during analysis was that certain fragment ions could be explained by multiple plausible hypotheses resulting from the co-existence of positional isomers of carbamoylation. For example, a y-ion of one positional isomer is indistinguishable by mass from a z-57 ion of a different positional isomer (figure 6). ECD produces ions with shifts of +57 Da for c ions and -57 Da for z ions diagnostic for isoaspartate, (figure 7). Therefore, careful manual examination of the assignments was imperative to differentiate the peaks corresponding to each positional isomer.

Peak corresponding to an "b" or "y" ion of one structure of a positional isomer are isobaric as peaks "c" or "z" (isoD) of other structures corresponding to other positional isomers.

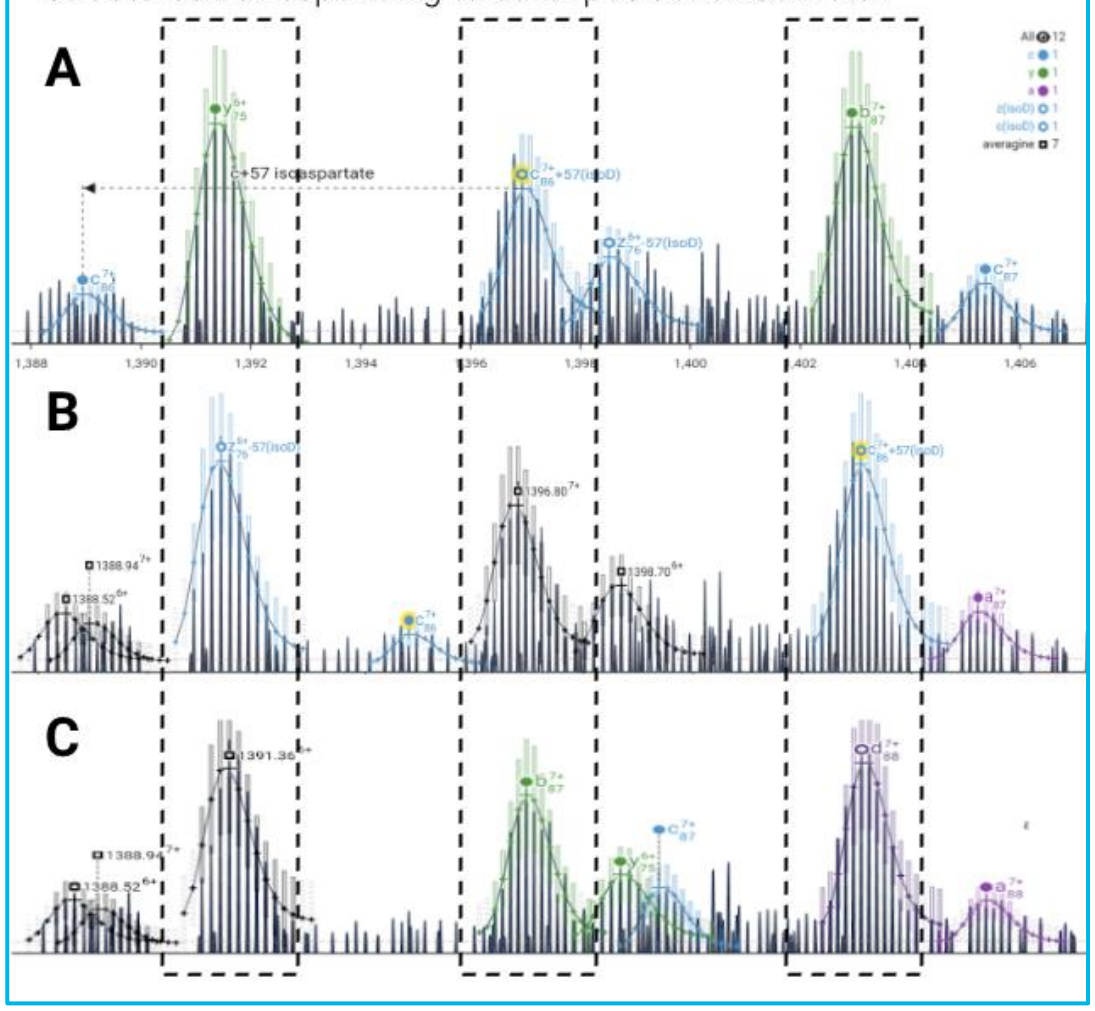

Figure 6. ECD selected spectrum of carbamoylated protein matched with 3 different isobaric sequences of positional isomers (A,B,C).

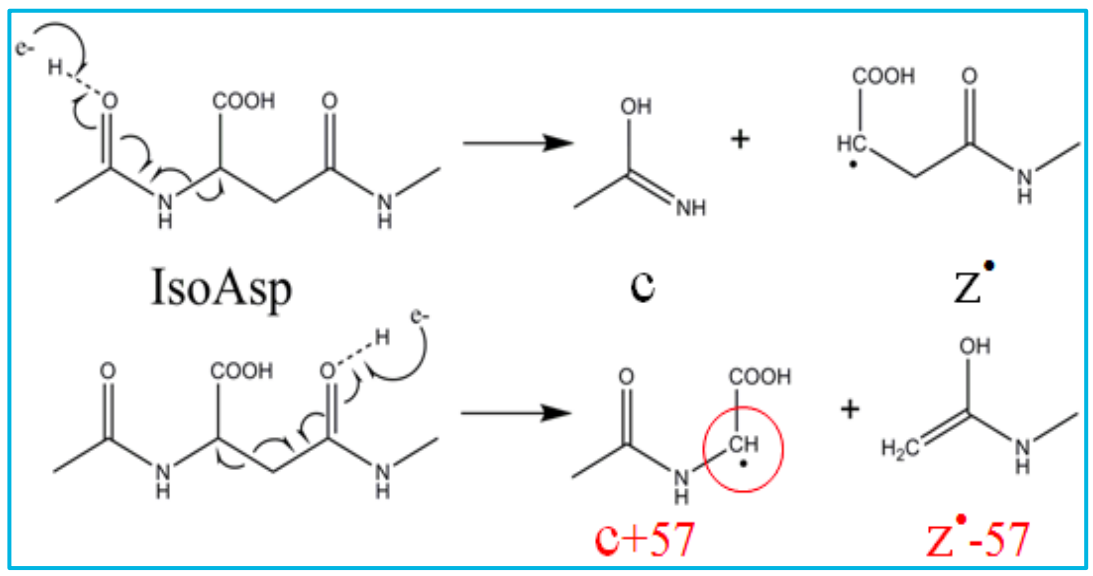

Figure 7. Isoaspartate c and z ions after ECD.

Carbamoylation site analysis

Through a comparative analysis of the distinct structures of carbamoylation isomers in ExDViewer, we have been able to establish the relative susceptibility of Lysine residues to carbamoylation by comparing their coverage results. Our findings reveal that Lysine residues located in close proximity to the N-terminal position were observed to undergo carbamoylation in nearly 90% of the protein species, whereas other residues were modified with significantly lower frequency. These results suggest a correlation between carbamoylation and protein folding, with the N-terminus being more exposed to the water-soluble cyanate.

Conclusions

ECD coupled to high resolution MS is a sensitive analytical approach to measure Lys carbamoylation associated with high urea concentration used for purification of therapeutic recombinant proteins.

References

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