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Localization of Disulfide Bonds in Ribonuclease Using Low pH Trypsin and LC-ESI-QTOF-MS

Amélie Bornex[a,b], and Saša M. Miladinović*[a]

*Correspondence: Prof. S. M. Miladinović, E-mail: sasa.miladinovic@hevs.ch
[a]HES-SO Valais-Wallis, University of Applied Sciences, Institute of Life Sciences, CH-1950 Sion; [b]Lonza AG, CH-3930 Visp

Abstract: We evaluated a method to localize disulfide bonds in bovine pancreatic ribonuclease (RNase) by applying low pH trypsin protein digestion with a bottom-up LC-ESI-QTOF-MS approach. The goal was to minimize disulfide bond scrambling during sample preparation. By using *N*-ethylmaleimide (NEM) for alkylation of free cysteines, we achieved 92% sequence coverage and successfully identified the native disulfide bonds between specific cysteine residues. However, scrambled disulfide bonds were also observed. Our results indicate that while this low pH digestion method helps preserve native disulfide bonds, further refinement is needed to fully prevent disulfide bond rearrangement during sample preparation.

Keywords: Bottom-up proteomics · Disulfide bonds · LC-MS · Low pH trypsin

Introduction

Proteins are biopolymers composed of amino acids linked by peptide bonds, folding into specific three-dimensional structures essential for their biological function. The stability of these structures is maintained by various non-covalent interactions and critical covalent disulfide bonds formed between the thiol groups of cysteine residues. Rearrangement of disulfide bonds, known as disulfide bond scrambling, can occur under certain conditions such as high pH or denaturing environments, potentially leading to loss of protein bioactivity. In protein-based therapeutics, correct disulfide bond formation is crucial, as mispairing can significantly affect efficacy and stability. Therefore, comprehensive characterization of disulfide bond patterns in proteins is essential.[1–3] However, sample preparation often employs high pH or relatively harsh conditions that could promote disulfide bond scrambling.[4,5]

The low pH trypsin kit was designed especially for disulfide bond analysis by bottom-up approach,[6] since it is supposed to prevent disulfide bond scrambling due to its low pH reaction buffer and proteases that were modified to be stable in low pH. The objective of the study is to develop a reliable approach for identifying disulfide bonds using liquid chromatography-electrospray ionization quadrupole-time-of-flight mass spectrometry (LC-ESI-QTOF-MS) while evaluating sample preparation techniques with low pH proteolytic digestion. *Bovine pancreatic ribonuclease* (RNase) was selected as the model protein due to its well-characterized structure (Fig. 1) and known four disulfide bonds, facilitating the validation of the methodology.

Method

A bottom-up proteomic approach was developed, involving the proteolytic digestion of RNase using a low pH trypsin kit designed to operate under acidic conditions, thereby reducing disulfide bond potential scrambling. The kit's protocol from Promega was followed, and samples were prepared under non-reducing conditions.[8] RNase samples were prepared by dissolving the protein in 8 M urea with 100 mM Tris buffer to denature the protein and expose cysteine residues. Free cysteines were alkylated using *N*-ethylmaleimide (NEM). The alkylation reaction was performed for 30 minutes in the dark at room temperature to prevent side reactions. Following alkylation, the sample was diluted, and proteolytic digestion was carried out using trypsin at an enzyme-to-protein ratio of 1:20. To stop the enzymatic reaction, formic acid was added to a final concentration of 1%. The sample was then filtered using centrifugal filters to remove any undigested material and subjected to a clean-up process using C18 solid-phase extraction to concentrate and desalt the peptides.

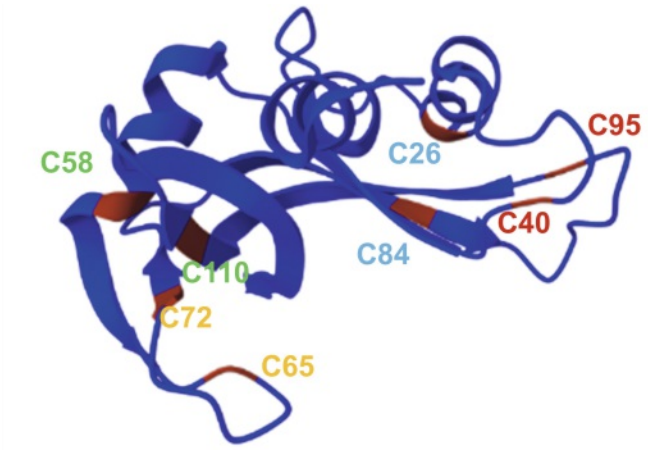

Fig. 1. 3D model of RNase with its natural disulfide bonds between Cysteines depicted with the same colour.[7]

The analysis was conducted using an Agilent 1260 HPLC employing C18 separation and Agilent QTOF 6550 mass spectrometer, allowing high-resolution detection of peptides and disulfide-linked peptides. The mass accuracy was maintained below 5 ppm with a resolution of 35'000. Data analysis was performed using Agilent's Bioconfirm software, focusing on the identification of alkylated and disulfide-bonded peptides.

Results

The comparative analysis of alkylation agents revealed that NEM provided superior results over iodoacetamide (IAM). In non-denaturing conditions, NEM produced a stronger MS signal and effectively alkylated free cysteines (Fig. 2).

Digestion with the low pH trypsin kit resulted in detection of disulfide-linked peptides. The low pH environment preserved the native disulfide bonds during digestion. The sequence coverage achieved was 92%, and several disulfide bonds were identified, including native bonds between cysteine residues C26–C84, C40–C95, C58–C110, and C65–C72 (Figs. 1 and 3). Additionally, scrambled disulfide bonds were detected, such as C26–C40 and C58–C84, indicating that rearrangement occurred, possibly during the sample preparation, despite the low pH conditions.

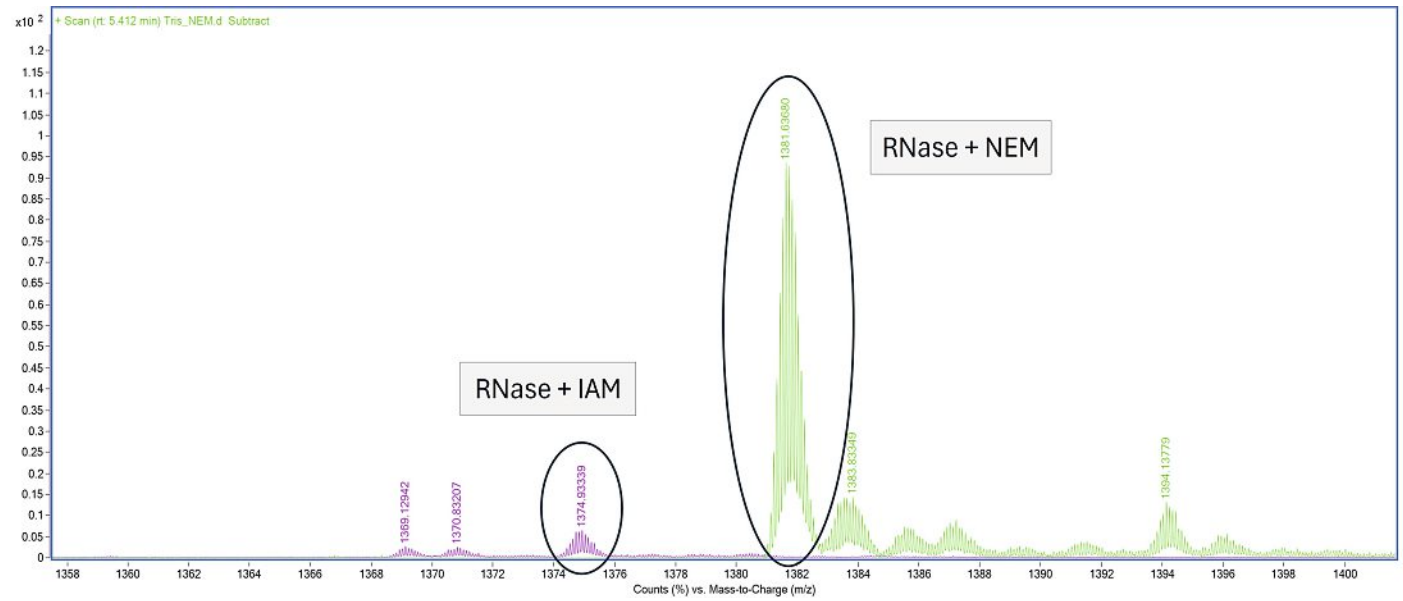

Fig. 2. Mass spectra comparison of alkylated RNase with NEM (Green) and IAM (Pink).

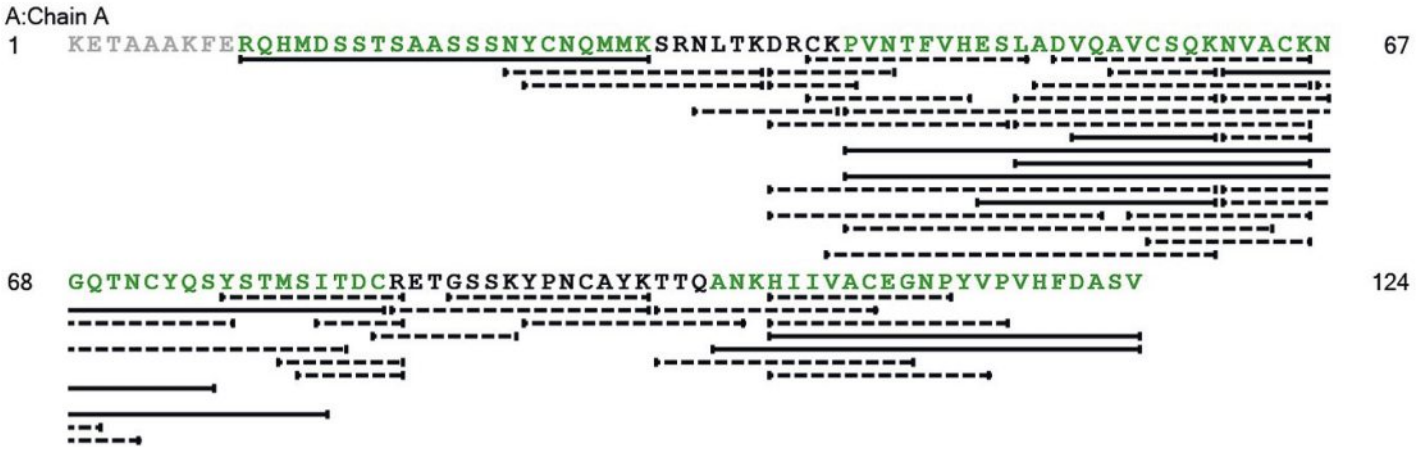

Fig. 3. Sequence coverage (93%) of the non-reduced sample. The dotted lines indicate disulfide bond connected peptides.

Conclusion

A method for localizing disulfide bonds in proteins was introduced in this study, utilizing low pH trypsin digestion and LC-MS analysis. While the results demonstrated the presence of native and scrambled disulfide bonds in RNase, further refinement of the methodology, particularly in sample preparation, is required. Future work should focus on using partial reduction techniques and exploring the use of alternative buffers to minimize disulfide bond scrambling. Overall, this research contributes valuable insights into the complex process of disulfide bond analysis in proteins and sets the stage for more advanced studies in this area.

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