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Short communication

m-nitrobenzyl alcohol supercharging reagent enhances the chromatographic separation and the charging of disulfide bond linked and His-tag peptides

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ABSTRACT

The linkages of disulfide bond (DSB) play important roles in protein stability and activity. Mass spectrometry-based (MS-based) techniques become accepted tools for DSB analysis in the recent decade. In the bottom-up approach, after enzyme digestion, the neighbouring amino acids of cysteines have great impacts on the physi-cochemical properties of resulting disulfide bond peptides, determining their retention behaviour on liquid chromatography (LC) and their MS ionization efficiency. In this study, the addition of supercharging reagent in LC mobile phase was used to examine the impact of supercharging reagent on the charge states of disulfide-bond peptides. The results showed that 0.1 % m-nitrobenzyl alcohol (m-NBA) in LC mobile phase increased the sensitivity and charge states of DSB peptides from our model protein, equine Interleukin-5 (eIL5), as well as the resolution of reversed-phase chromatography. Notably, also the sensitivity of C-terminal peptide with His-tag significantly improved. Our findings highlight the effectiveness of employing m-NBA as a supercharging re-agent when investigating disulfide-linked peptides and the C-terminal peptide with a His-tag through nano-liquid chromatography mass spectrometry.

1. Introduction

The structure of proteins is intricately determined not only by their amino acid sequence but also by the folding process, which includes the formation of disulfide bonds (DSB). These bonds, created between the sulphur atoms of two cysteine residues, play a critical role in stabilizing the three-dimensional structure of proteins. Thus, it is important to characterize DSB to ensure the quality and function of protein phar-maceuticals. Thanks to the developments in proteomics, mass spectrometry-based (MS-based) techniques are now wildly accepted tools for DSB analysis [1]. The surrounding sequences of cysteines have great impacts on the physical and chemical properties of resulting di-sulfide bond-linked peptides after enzyme digestion. Hence, the length of peptide sequences will determine the retention behaviour on liquid chromatography (LC) and ionization efficiency of the resulting disulfide-linked peptides on LC-MS setup. It is very often that the larger disulfide-linked peptides could not be detected by only one enzyme approach due to the size of DSB-linked peptides and MS limitation on

lower sensitivity on the higher mass range [2]. To reduce the length of disulfide-linked peptides and increase the coverage of DSB mapping, multi-enzyme digestion is commonly used in this type of analysis [2,3]. However, it is not applicable when the sample amount is limited, and the data analysis becomes more complicated when a multi-enzyme approach is applied. For these reasons, there is a constant need for new analytical approaches improving the mapping of DSB peptides without increasing costs or complexity.

The term "supercharging" was first invented by Williams and co-workers to describe the increased charging observed in spectra ob-tained from solvents supplemented with a variety of chemicals [4]. The presence of supercharging reagents reduces mass-to-charge (m/z) re-quirements for the mass analysers, improves mass resolution and mass accuracy, enhances MS/MS fragmentation, and increases sequence coverage [5]. Among them, m-nitrobenzyl alcohol (m-NBA) and dimethyl sulfoxide (DMSO) are well-known to enhance the charge states (z) of macromolecules, i.e., protein and peptides [6–8]. In our previous study, we showed that the presence of m-NBA in LC mobile phase not

only improved the chromatographic separation but also increased the charge states of glycopeptides with higher molecular weight [9]. The same effect was also reported when m-NBA was used to reduce the ion suppression caused by trifluoroacetic acid (TFA) in the common mobile phase for reversed-phase chromatography [5].Hence, we proposed that the presence of m-NBA in the LC mobile phase could improve the chromatographic resolution of disulfide-linked peptides on reversed-phase separation and increase the charge stats of DSB peptides.

We tested this hypothesis in the context of insect bite hypersensitivity (IBH), a type I/IVb hypersensitivity reaction triggered by *Culicoides* bites. The proteins present in *Culicoides*' salivary glands have the potential to induce IgE production, activate mast cells and basophils, and cause the accumulation of eosinophils in the skin during the late phase of type I and delayed type IVb hypersensitivity reaction [10,11]. Interleukin-5 (IL-5) plays a crucial role in the differentiation, activation, and survival of eosinophils [12]. Recent studies have demonstrated that targeting IL-5 could be an effective approach in the treatment of horses affected by IBH. Fettelschoss-Gabriel A. *et al.* showed IL-5 to be a suitable target for treating IBH-affected horses and developed a therapeutic vaccine consisting of equine IL-5 (eIL-5) linked to virus-like particles (VLPs) [13–15]. The endogenous eIL-5 (*Equus caballus*; Uniprot O02699) contains two cysteines that may form a DSB (C45 and C87). To ensure the free cysteine(s) for VLP conjugation, we introduced a third cysteine at the C-terminal before His-Tag (C119). Interestingly, our data showed that the recombinant eIL-5 has higher tendency for dimerization after purification [13]. Thus, the formation of disulfide bonds and the presence of free cysteine(s) are crucial for the efficiency of VLP conjugation to eIL-5, which was used for this study.

2. Experimental section

2.1. eiF5 expression and purification

Recombinant eIL-5 was expressed in *Escherichia coli* BL21 (DE3) cells, isolated from inclusion bodies, purified, and concentrated over an IMAC chromatography (HisPrep FF 16/10, Cytiva), rapid refolded overnight, and polished with a size-exclusion chromatography (Superdex 75 26/600, Cytiva) to separate the dimer from multimers.

2.2. Sample preparation and nanoUPLC-MS/MS analysis

Ten micrograms of eIL5 were digested with 0.2 μg of trypsin in 50 mM sodium acetate buffer (pH 6) with and without previous alkylation using 5mM N-ethylmaleimide (NEM, Sigma). NEM was used to identify the free cysteines and prevent the scrambling of DSB [16]. After overnight digestion, both samples were dried by SpeedVac (Thermo Scientific), dissolved in 3 % acetonitrile (ACN) with 0.1 % formic acid (FA), and divided into three aliquots. The same amount of each aliquot was injected on a nanoAcquity system (Waters) coupled to a Q Exactive™ mass spectrometer (Thermo Fischer) equipped with a Picoview™ nanospray source 500 (New Objective). Samples were loaded onto a Symmetry C18 trap column (180 μm × 20 mm, 100 Å, 5 μm particle size) and separated on a HSS T3 C18 column (75 μm × 250 mm, 100 Å, 1.8 μm particle size), at a constant flow rate of 300 nL/min, with a column temperature of 50°C. The three different combinations of mobile phase

were tested in this study are shown in Table 1.

For 0.1 % FA mobile phase condition (1), peptides were eluted with a gradient from 5 to 35 % B in 62 min, 35 to 60 % B in 10 min, 60 to 95 % B in 10 min, held isocratically at 95 % B for another 10 min before equilibrating back to 5 % B. The gradient of 0.1 % m-NBA and 0.5 % m-NBA was adjusted to the same concentration of acetonitrile as the condition containing only 0.1 % FA. Before the measurement, the LC system was flushed with the testing mobile phase condition and equilibrated overnight and the LC gradient in each measurement was adjusted to the same percentage of ACN. For MS setting, one scan cycle comprised of a full scan MS survey spectrum, followed by HCD (higher-energy collision dissociation) fragmentation on the 12 most intense signals per cycle. Both MS and MS/MS scans were measured by Orbitrap mass spectrometer. Full-scan MS spectra (300–2'000 m/z) were acquired at a resolution of 70'000 at 400 m/z, while HCD MS/MS spectra were acquired at a resolution of 35'000. HCD MS/MS spectra were performed with a target value of 1e5 using a normalized collision energy 25 %. The samples were acquired using internal lock mass calibration on m/z 371.1010 and 445.1200.

2.3. Data analysis

All raw data were search against the database eIL5 sequence using Byonic v4.6 (Protein Metrics, USA) search engine including oxidation of methionine and alkylation of cysteines using NEM. For plotting the eXtracted Ion Chromatograms (XICs) and peak area, Skyline (23.1.0.268) was used [17].

3. RESULTS

3.1. eIL5 contains both inter- and intramolecular disulfide bonds

The LC-MS/MS peptide spectrum match (PSM) results from all experiments showed the formation of disulfide bonds between C45-C45, C45-C87, and most likely, C119 was not involved in disulfide bond formation. According to the number of PSMs, the major form was C45-C87 DSB and C45-C45 was minor form (Table 2, Supplementary Fig. 1). Based on size-exclusion chromatography results, the major form of eIL5 is a dimer (Supplementary Fig. 2). Therefore, the results suggested that C45-C87 bond was intermolecular instead of intramolecular. In addition, the cysteines with NEM were observed in all three sites, suggesting three types of dimers in the sample: (a) double C45-C87 and C87-C45 DSB, (b) single C45-C87 DSB, (c) single C45-C45 DSB. C119 was found free (Supplementary Fig. 1).

3.2. m-NBA supercharging reagent improves the detection of DSB-linked peptides

The signals from eIL5 DSB peptides could be observed when using 0.1 % FA condition (1), however, the peak width of C45-C45 linked peptides was broad, eluting over 5 minutes (Fig. 1). This might be due to the strong hydrophobic interaction between DSB-linked peptides and reversed-phase materials. In our previous study, we observed that the supercharging reagent not only help on increasing the charge states of

Table 1
Mobile phases A and B used in the present study.

#	Mobile phase condition	Mobile phase A	Mobile phase B
(1)	0.1 % FA	0.1 % FA in water	0.1 % FA in ACN
(2)	0.1 % m-NBA	0.1 % FA + 0.1 % m-NBA in 4.8 % ACN	0.1 % FA + 0.1 % m-NBA in 95 % ACN
(3)	0.5 % m-NBA	0.1 % FA + 0.5 % m-NBA in 4.4 % ACN	0.1 % FA + 0.5 %m-NBA in 95 % ACN

Table 2
The list of observed eIL5 DSB peptides. PSM = peptide-spectrum match. The data was from Byonic search results and filtered based on following criteria: score > 150, deltamodscore > 10; mass error < 3 ppm, only peptides that show specific trypsin cleavage were considered.

Disulfide bond peptides	Amino acid position	# of PSM
NHQL[45]CIEEVFQGIDTLK - NHQL[45]CIEEVFQGIDTLK	C45-C45	19
NHQL[45]CIEEVFQGIDTLK - KK[87]CGGER	C45-C87	25
NHQL[45]CIEEVFQGIDTLK - K[87]CGGER	C45-C87	362
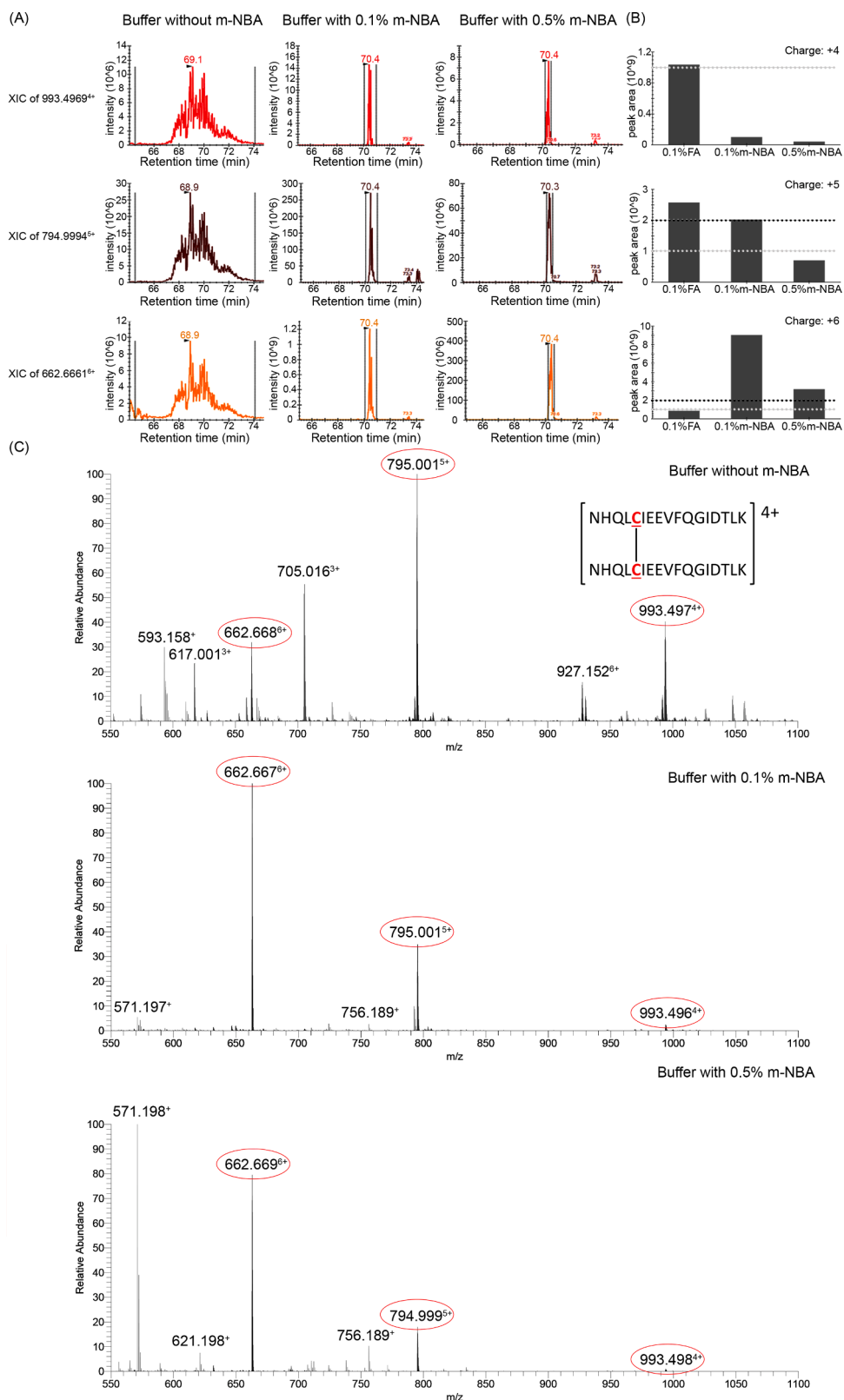
Fig. 1. The effect of m-NBA on disulfide-linked peptides on chromatography and charge states. Extracted ion chromatograms (XIC) of disulphide-bond linked peptides from eIL-5 under different mobile phase conditions. The XIC of +4 to +6 from C45-C45 linked tryptic peptides were shown with the mass tolerance 10 ppm (A), peak area of charge states from +4 to +6 (B) and MS spectra from corresponding conditions (C). The *y*-axis of XIC and MS spectra was absolute intensity and relative intensity, respectively. The m/z with red circle represented the C45-C45 peptides with different charge states. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glycopeptides but also improved the chromatography on reserve phase separation [9]. Thus, we proposed that m-NBA would also improve the charge states and chromatography of DSB-linked peptides. When using m-NBA, the results showed that the most intense charge state of C45-C45 linked peptides, NHQL^{45}CIEEVFQGIDTLK-NHQL^{45}CIEEVFQGIDTLK, increased from z=+5 to z=+6. The intensity of z=+6 increased 3 folds with 0.1 % m-NBA (2) and 5 folds with 0.5 % m-NBA (3), meaning that the m-NBA facilitated the transfer of protons to DSB-linked peptides during ionization (Fig. 1A). Moreover, the chromatographic resolution of these DSB-linked peptides significantly improved with the presence of m-NBA, full width at half maximum (FWHM) reducing from 2.5 min in 0.1 % FA (1) to 0.14 or 0.16 min when the mobile phase contained 0.1 % m-NBA or 0.5 % m-NBA. The peak area of each charge state showed the significant enhancement of DSB peptide with six protons on MS detection when the mobile phase contained 0.1 % m-NBA (Fig. 1B). In addition, the major co-eluted peptides, m/z 705.016 and m/z 927.152, were eluted later than C45-C45 linked DSB peptides and the charge states increased as well (Fig. 1C). We observed the same trend on the C45-C87 linked peptide (NHQL^{45}CIEEVFQGIDTLK-KK^{87}CGGER and NHQL^{45}CIEEVFQGIDTLK-K^{87}CGGER, Supplementary Fig. 3). Our results showed that m-NBA could increase not only the charge state of DSB peptides but also the sensitivity. In addition, the data also showed the improvement of resolution on reversed-phase chromatography when m-NBA was applied.

3.3. m-NBA supercharging reagent improves the detection of C-terminal peptide with His-Tag

Through our studies, the C-terminal peptide with His-Tag was not consistently observed. However, the results of intact protein mass determination showed that the molecular weight of eIL-5 was intact without any truncation (data not shown). Therefore, we further investigated the effects of supercharging reagent on the C-terminal peptide with His-Tag, QFLDYLQEFLGVINTEWTIEGGGCHHHHHH-. In this analysis, the tryptic C-Terminal peptide was not observed in 0.1 % FA condition (1). Interestingly, the peptide was well detected at retention time 78-79 min when the mobile phase contained m-NBA (Fig. 2). The

high intensity precursor ions allowed to obtain good quality MS/MS spectra, confirming the identity of the C-terminal peptide from eIL-5 (Supplementary Fig. 4).

3.4. High concentrations of m-NBA have negative effects at a global peptide level

To understand the effects of supercharging reagent on a larger scale analysis, the individual charge states from total MS/MS spectral counts and PSMs were analysed (Fig. 2). The highest charge state detected in 0.1 % FA condition (1) was z=+6, z=+14 in 0.1 % m-NBA (2), and z=+12 in 0.5 m-NBA (3). In addition, 20 to 23.7 % of PSMs were identified above z=+5 in 0.1 % m-NBA (2) and 0.5 % m-NBA (3), while only 10 % was identified in 0.1 % FA (1). These results indicated that the supercharging reagent facilitated the transfer of protons to peptides. However, we observed a 57 % reduction on the number of PSMs in 0.5 % m-NBA (3), from 1381 to 591. Besides, the total ion chromatography (TIC) signal in 0.5 % m-NBA significantly decreased, indicating a potential ion suppression at higher supercharging reagent concentration. For this reason, the mobile phase containing 0.1 % m-NBA (2) was considered as the ideal condition for DSB analysis (Fig. 3).

4. Discussion

Thanks to the developments in mass spectrometry, MS-based proteomic methods are widely used for the characterization of biomolecules. In the proteomic landscape, the most common strategy is to digest proteins into peptides using enzymes such as trypsin, so that most peptides are in the favourable m/z range of mass analysers. In addition, the front-end liquid chromatography is also optimized for the separation of tryptic peptides. However, this strategy has its limitation for broader applications. Among them, major limitations are the proton accessibility for lower abundant and larger peptides during ionization, and the range of m/z detection of mass analysers. There are two ways to influence the number of protons carried per molecule to improve the detection on MS analysis. Proton transfer charge reduction (PTCR) utilizes ion–ion proton transfer reaction to reduce the charge states of proteins, shifting the
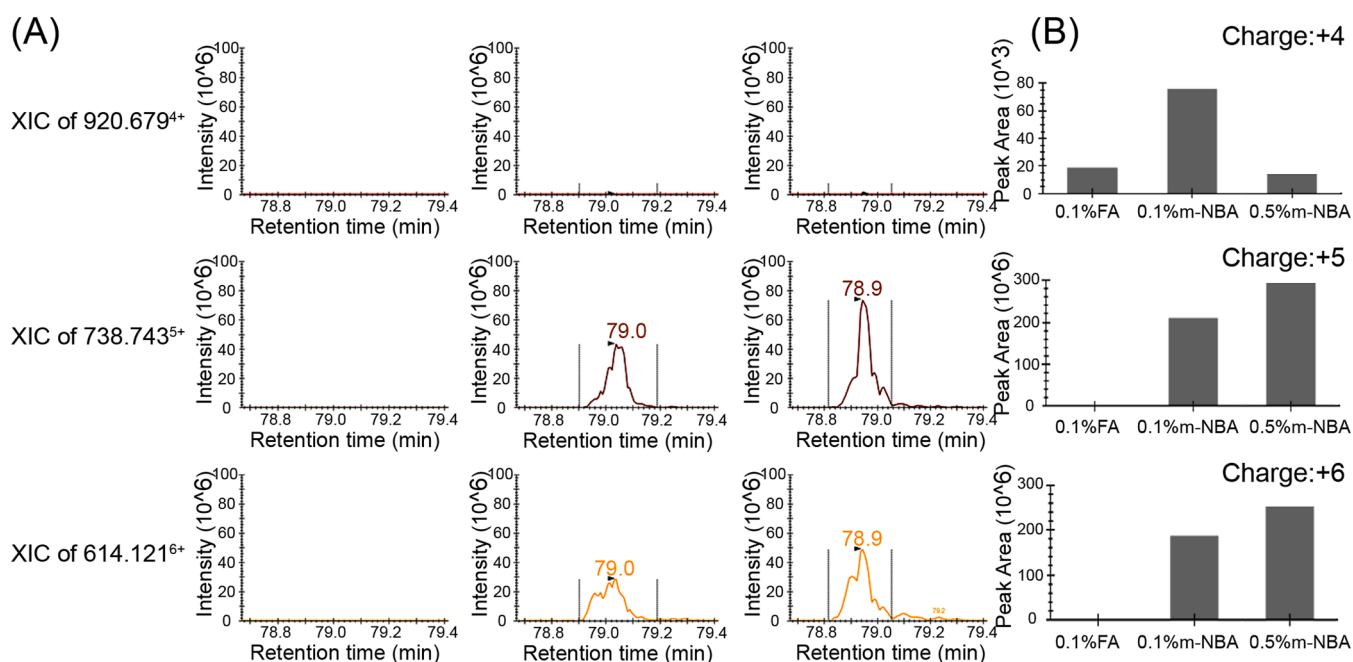

Fig. 2. The effect of m-NBA on C-terminal peptide with His-Tag on chromatography and charge states. Extracted ion chromatography (XIC) of disulphide-bond linked peptides from eIL-5 under different mobile phase conditions. The XIC of +4 to +6 from QFLDYLQEFLGVINTEWTIEGGGCHHHHHH- were shown with the mass tolerance 10 ppm (A). The *y*-axis was absolute intensity. (B) Peak area of charge state from +4 to +6.
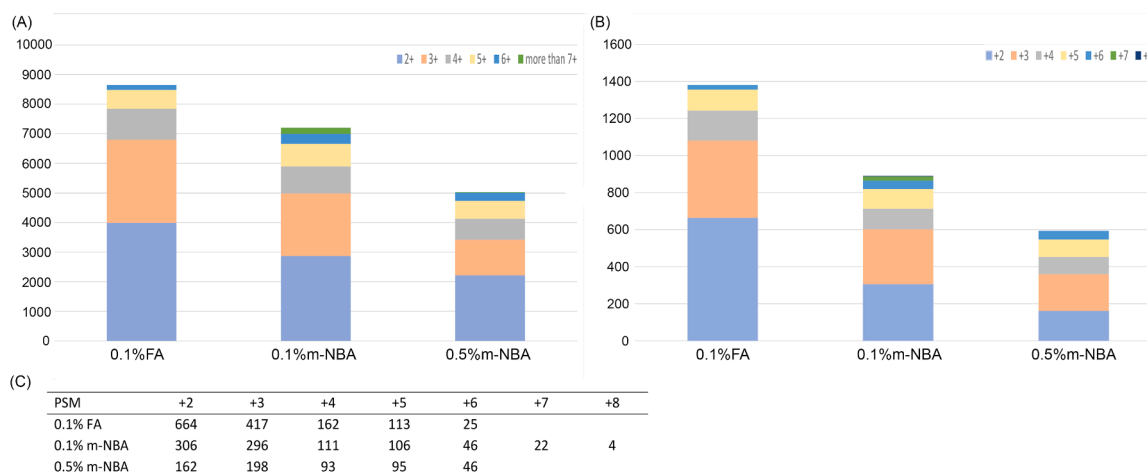
Fig. 3. Distribution of charge states in each chromatographic condition. Sum of MS/MS spectra (A) and peptide-spectrum matches (PSMs, B) from the charge states indicated in the chart. The detailed PSMs results are shown (C).

protein signals to higher m/z range and preventing the overlay with signals from small molecules in the lower m/z range [18]. The current main application of PTCR is top-down proteoform analysis [19]. The opposite approach to steer the number of protons per molecule is to increase the charge states of biomolecules with the help of supercharging reagents [8].

In this study, the results showed that both charge states and signal intensities of DSB-linked peptides of protein eIL5 increased in the presence of the supercharging reagent m-NBA. The improved quality of the MS/MS spectra could provide solid proof of C45-C45 and C45-C87 linkage for the eIL-5 protein. Interestingly, we also observed that the effects of m-NBA supercharging reagent on two DSB linkages were not equal, indicating that the proton accessibility could be sequence-dependent, or structure-related as described by Going *et al.* [20]. The signals of the C-terminal peptide containing an His-Tag were also increased when the mobile phase contained m-NBA. This showed that m-NBA could facilitate the protons on the His-rich C-terminal peptide. These results indicate that the most common used mobile phase for proteomic purposes is suited for most tryptic peptides but cannot cover the complexity of all digested peptides.

In the presence of m-NBA, the resolution of DSB peptides on reversed-phase column was also enhanced, as we have previously shown for glycopeptides [9]. This may be explained by a reduction of surface tension between peptides and solid phase caused by m-NBA. Another hypothesis is that the combination of m-NBA and formic acid acts similarly to an ion-pairing reagent, protecting cationic species from interacting with the negatively charged silanols of the column to maintain pseudoneutrality. This may reduce solute band broadening, resulting in narrower chromatographic peaks. However, we observed a significant decrease of PSMs using 0.5 % m-NBA condition (3) although the intensity signals of DSB were the highest. In addition, the intensity of total ion chromatography was also decreased. These results suggests that potential ion suppression occurs during ionization when the concentration of m-NBA is too high.

Charge manipulation is not the only strategy to improve the identification of DSB peptides. Ion mobility spectrometry (IMS) can also be used to enhance signal-to-noise (S/N) ratios by separating ions with different mobilities prior to mass analysis. High-field asymmetric waveform ion mobility spectrometry (FAIMS) has been applied already on many applications, including the analysis for intact protein assemblies and protein complexes [21], showing the potential for disulfide bond analysis on other instrumental setups. However, both PTCR and FAIMS analysis are only available on certain types of instruments. Despite the usage of 0.1 % m-NBA was not tested for the analysis of other proteins, the addition of supercharging reagent can be easily

implemented in any laboratory at very low cost and could potentially increasing the sequence coverage for MS-based analysis of DSB-linked peptides, and without requiring any extra device such as FAIMS or PTCR.

5. Conclusions

The formation of DSB on eIL5 was characterized in this study. The major disulfide bonds were between C45-C45 and C45-C85. The free cysteine at position 119 was confirmed in this study and showed the accessibility for further conjugation. In addition, the presented study provides a novel analytical approach to increase the charge states and chromatography of DSB peptides by combining the application of the supercharging reagents m-NBA with a state-of-the-art nanoUPLC−ESI-MS/MS method. The presence of 0.1 % m-NBA supercharging reagent into the LC mobile phases not only significantly improved the chromatographic behaviour but also the MS1 signal intensity of DSB peptides and C-terminal peptide with His-Tag. Hence, it could lead to the better assignments on MS2 spectra. The concentration of m-NBA was optimized for this specific protein, suggesting that an optimization might be required for each experiment. The ion suppression was observed when the concentration of m-NBA reached 0.5 %. This was consistent with our previous study [9]. It indicates that the fine tuning of m-NBA concentration for each application is crucial. The data showed that the addition of m-NBA could not only increase the coverage of protein but also the confirmation of DSB peptides.

CRediT authorship contribution statement

Chia-wei Lin: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Fabia Canonica:** Conceptualization. **Simone Wüthrich:** Methodology, Formal analysis. **Antonia Fettelschoss-Gabriel:** Conceptualization. **Ralph Schlapbach:** Resources, Funding acquisition. **Paolo Nanni:** Writing – review & editing, Supervision, Project administration, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.464828.

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