


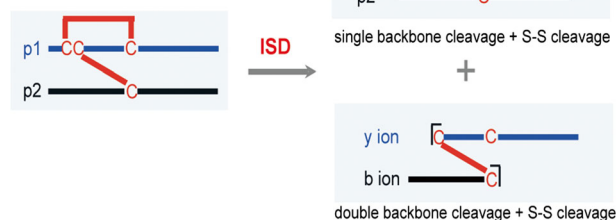
RESEARCH ARTICLE

Characterization of Protein Disulfide Linkages by MS In-Source Dissociation Comparing to CID and ETD Tandem MS

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Insulin peptide intra- and inter-chain S-S linkages



Abstract. Direct characterization of disulfide linkages in proteins by mass spectrometry has been challenging. Here, we report analysis of disulfide linkages in insulin variant, endothelin 3, and relaxin 2 by in-source dissociation (ISD) during LC-MS. A duplet insulin peptide from Glu-C digestion that contains peptides p1 and p2 (from chains A and B, respectively) was selected as a model peptide. This duplet peptide has an inter-chain disulfide bond between p1 and p2, and an intra-

chain disulfide bond in p1. To compare the gas-phase fragmentation, it was subjected to ISD MS and MS/MS methods, including collision-induced dissociation (CID) and electron transfer dissociation (ETD). The pattern and efficiency of peptide backbone and disulfide cleavage varied with these dissociation methods. ETD, CID, and ISD were able to generate single backbone, double backbone, and triple (double backbone and single disulfide bond) cleavages in this model peptide, respectively. Specifically, CID did not cleave disulfide bonds and ETD was able to only cleave the inter-chain disulfide bond at low efficiency, limiting their usage in this disulfide analysis. In contrast, ISD was able to cleave the intra-chain disulfide bond in addition to peptide backbone, creating multiple fragment ions that allow accurate assignment of both intra- and inter-chain disulfide linkages. ISD was also successfully applied to determine double disulfide linkages in endothelin 3 and relaxin 2 peptides. This study contributes to the fundamental understanding of disulfide bond cleavages in different gas-phase fragmentations and provides an efficient cleavage strategy for identification of disulfide bonds in proteins by ISD ESI-MS.

Keywords: Disulfide bond, Insulin, Relaxin 2, Endothelin 3, In-source dissociation (ISD), LC-MS, CID, ETD, MS/MS, Glu-C digestion, Trypsin and Lys-C digestion

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Introduction

Disulfide linkages are critical for the overall structure stability of many proteins and their biological activities [1]. Insulin is a hormone produced by the pancreas and plays a critical role in regulating blood glucose levels in human via inhibition of gluconeogenesis [2, 3]. It is a small protein with a molecular mass of ~ 5800 Da, consisting of two peptide chains

(chains A and B) linked by two inter-chain disulfide bonds with one intra-chain disulfide bond in chain A [4]. A large group of insulin-related small proteins including insulin, insulin-like growth factor (IGF), relaxin sub-family, and insulin-like peptides have been identified. They all have the conserved six cysteine residues connected by two inter-chain disulfide bonds and one intra-chain disulfide bond within chain A [5].

Multiple forms of insulin including human insulin, insulin analogs, and insulin biosimilars are developed in pharmaceutical industry for treatment of diabetes [6]. Structurally similar to insulin, relaxin, also a small peptide hormone, plays a role in female reproductive system [7, 8]. Endothelins are vasoconstrictive single chain peptides with two disulfide bonds and are primarily produced in the endothelium with an

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important role in vascular homeostasis [9, 10]. Disulfide bond characterization is important for understanding manufacturing process consistency and product quality control of various insulin and relaxin drugs.

Currently, most disulfide bond linkages are characterized by various partial reduction peptide mapping methods [11–14]. However, direct characterization of disulfide bonds by mass spectrometry (MS) is more desired although it is very challenging. Two major types of gas-phase dissociation methods in MS are low-energy collision-induced dissociation (CID) and electron capture/transfer dissociation (ECD/ETD). They have been used to characterize the gas-phase fragmentation of disulfide bond-containing peptides or proteins [15]. Low-energy CID normally only cleaves the peptide backbone but not disulfide bonds, because higher dissociation energy is required for disulfide bond cleavages [16]. Therefore, many strategies have been developed to improve disulfide bond dissociation by CID. They include metal ion assisted fragmentation [17, 18], methoxy addition with UV irradiation [19], super-charging prior to MS analysis [20], in-source reduction [21, 22], electrochemical reduction of disulfide bonds [23–29], plasma-induced disulfide bond cleavage [30], generation of dehydroalanine for selective cleavage [31], and negative mode dissociation followed by MS [32]. ECD was reported to preferentially cleave disulfide bonds [33]. ETD, as an analog to ECD, is also widely used to elucidate disulfide bonds [34, 35]. However, the efficiency of disulfide cleavage by ECD and ETD is greatly impacted by the composition, size, and charge of peptides [36–38].

In-source dissociation (ISD) of ions generated by electrospray ionization (ESI) occurs in a relatively high pressure region during the transfer of the ions from the source to the vacuum chamber of a mass spectrometer. ISD allows ion population in all charge states for fragmentation without precursor isolation compared to CID and ETD, and therefore generally creates higher intensity of fragmentation signals than latter methods. However, thus far, ISD has been a much less reported application than CID and ECD/ETD [39].

In this work, a duplet insulin peptide containing an inter-chain and an intra-chain disulfide bonds was used to directly characterize the disulfide linkages by different MS dissociation methods. Unlike ETD and CID, ISD induced single- or double-backbone cleavages as well as disulfide cleavage, and thus provided a more powerful but simple tool to elucidate inter- or intra-chain disulfide bond linkages. The ISD method was also used to elucidate disulfide linkages in endothelin 3 and relaxin 2 peptides.

Chemicals and Experiments

Chemicals and Reagents

Ammonium acetate and acetic acid were obtained from Sigma. Ethylenediaminetetra acetic acid (EDTA) (0.5 M, pH 8.0) was purchased from Promega. Tris(2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) and trifluoroacetic acid (TFA) were

from Thermo Scientific. Endoproteinase Glu-C enzyme (sequencing grade) was from Roche. Trypsin and Lys-C enzymes were from Promega and Wako, respectively. Mobile phases of 0.1% formic acid in water (Optima LC/MS grade) and 0.1% formic acid in acetonitrile (Optima LC/MS grade) were from Fisher Chemical. Pierce LTQ Velos ESI positive ion calibration solution was from Thermo Scientific.

Recombinant Human Insulin (RHI) Variant Sample, Endothelin 3, and Relaxin 2

The recombinant human insulin (RHI) sample was expressed in *Escherichia coli* and purified by multiple steps of chromatography at Merck & Co., Inc., Kenilworth, NJ, USA. Endothelin 3 was obtained from Sigma, and relaxin 2 was purchased from Phoenix Pharmaceuticals.

Non-reduced Protein Digestion

Insulin sample was digested by Glu-C. The digestion buffer was made by mixing 100 μ L of 100 mM ammonium acetate (pH \sim 5.0) and 40 μ L of 100 mM EDTA (pH 8.0). Insulin sample (100 μ g) was digested in this digestion buffer by Glu-C enzyme (5 μ g) in a total volume of 200 μ L at 37 $^{\circ}$ C for 4 h with shaking at 300 rpm on a thermomixer. Digestion reaction was quenched by adding 5 μ L of 20% TFA. For LC-MS analysis, 10 μ L of the digested sample was diluted by mixing with 90 μ L of 0.05% TFA.

Relaxin 2 was prepared in water at a final concentration of 2 mg/mL; 10 μ L of relaxin solution was mixed with 25 μ L of 8 M urea in 50 mM ammonium bicarbonate (pH 8.0), and incubated at 37 $^{\circ}$ C for 30 min, followed by adding 70 μ L of 50 mM ammonium bicarbonate. Denatured relaxin was digested by trypsin and Lys-C at 37 $^{\circ}$ C for 5 h at a 1:10 enzyme/substrate ratio for each enzyme. The reaction was terminated by adding 2.5 μ L of 20% TFA. Endothelin 3 was dissolved in water to a final concentration of 0.5 mg/mL and directly analyzed by LC-MS without digestion.

Reverse Phase LC-MS/MS

The digested and diluted insulin sample (1–1.5 μ g) was separated on a HALO peptide ES-C18 column (2.1 \times 150 mm, 2.7 μ m, Advanced Materials Technology) at a flow rate of 0.4 mL/min on a Waters ACQUITY H-class UPLC system. The column temperature and auto-sampler were set at 60 $^{\circ}$ C and 5 $^{\circ}$ C, respectively. Mobile phase A was 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. The mobile phase gradient was set as follows: 0–1 min 13% B, 3–6 min 17%–20% B, 9–13 min 26%–28% B, 15–17 min 95% B, 18–25 min 13% B. A Thermo Fisher LTQ Orbitrap Velos mass spectrometer was connected with the Waters UPLC system for LC-MS/MS analysis. Heated ESI source settings were as follows: source heater temperature at 300 $^{\circ}$ C, sheath, auxiliary, and sweep gases flow rate at 35, 10, and 0 arbitrary unit (arb), respectively, positive polarity, source voltage at 4.00 kV, capillary temperature at 350 $^{\circ}$ C, S-Lens RF level at 50.0%. For

MS and ISD MS, scan settings were as follows: FTMS analyzer, normal mass range, resolution at 30000, full scan type, profile data type. For CID and ETD MS/MS analysis, the parent ion was isolated in the orbitrap and fragmented at optimized normalized collision energy and activation time, respectively. Reagent ion used in ETD was fluoranthene. For ISD, the source fragmentation was on and the energy was optimized. Data were analyzed manually by using Xcalibur 4.0, GPMAW 10.0, and ChemDraw 15.0. The similar LC-MS ISD condition to insulin sample was used for endothelin 3 and relaxin 2 samples as described above.

Results and Discussion

Insulin, relaxin, and endothelin were used in this study as model molecules for characterization of disulfide linkages. Their amino acid sequences and structures are shown in Table 1.

Non-reduced Peptide Mapping of Recombinant Human Insulin

The peptides from the recombinant human insulin following Glu-C digestion under non-reducing condition were analyzed by reverse phase LC-MS, and four major peptides were identified. The total ion current (TIC) chromatogram is shown in Figure 1. Peak 1 corresponds to B(22–29) of chain B. All peptides in peaks 2–4 are duplet peptides linked by one inter-chain disulfide bond. Peak 2 represents A(19–22)/B(14–21). Peak 3 is A(6–18)/B(1–13) and Peak 4 is A(1–18)/B(1–13). The duplet peptide product in Peak 3 contains two short peptides called p1 and p2 (from chains A and B, respectively) that are linked by an inter-chain disulfide bond and has one intra-chain disulfide in p1. This duplet peptide was used as a model peptide to characterize both inter- and intra-chain disulfide linkages by ISD and MS² experiments.

Characterization of Double Disulfide Bonds in Insulin Peptide by CID, ETD, and ISD

CID Fragmentation

The m/z 743.08 (4+) ion, the most abundant precursor ion of this duplet peptide, was selected for targeted MS² by CID. The spectrum is shown in Figure 2a and detailed peak list is in supplemental Table S1. The peptide backbone cleavage pattern containing b and y ions is shown in inset of Figure 2a. In this MS² spectrum, major fragment ions were in 3+ and 2+ charge states. Most 3+ charged b ions (b_9 - b_{12}) were derived mainly from a single-backbone cleavage in p1 containing the intra-chain disulfide bond, and less from cleavage in p2 (Figure 2b). Therefore, the CID cleavages occur mostly on the p1 peptide and much less on p2. This is unexpected since the p2 peptide does not contain an intra-chain disulfide bond and should confer a more open structure for the cleavage. This preferential cleavage may be due to the higher basicity of p2 than p1 [40–42].

Interestingly, the ions from double cleavage of the backbones (one cleavage on each peptide of the duplet peptide), for example, (p1) b_{10} /(p2) b_{12} , (p1) b_{11} /(p2) b_{12} , and (p1) b_{12} /(p2) b_{11} , were also observed, albeit at a lower intensity (Figure 2b). The major 2+ fragment ions were the double-backbone cleavage products (Figure 2c). They are mainly b/b ions, (p1) b_{8-12} /(p2) b_{8-12} , and a few b/y and a/y ions including (p1) b_{12} /(p2) y_7 and (p1) a_{12} /(p2) y_{12} . The cleavages on side chains or disulfide bonds were not observed.

Although a single peptide backbone cleavage is common during CID, few reports have described the double-backbone cleavage of disulfide bond-containing peptides by CID [43]. These, however, did not specify whether it was in a single peptide chain or in both peptides. The current software for disulfide bond MS/MS analysis is mainly based on single backbone cleavage. Therefore update of analytical software with the double-backbone cleavage data for CID should be

Table 1. Amino Acid Sequence and Structure of Proteins Used in This Study

| | | |
|-----------------|--|---|
| Insulin variant | chain A | XXXXXXQ CCTSI C SLYQ LE XXX CX |
| | chain B | FVNQHLCGSHLVE XXXXXX CXXXXXXXXXX |
| Relaxin 2 | chain A | QLYSALANK CCHVG CTKRSLARFC |
| | chain B | DSWMEEVIKLCGRELVRAQIAICGMSTWS |
| Endothelin 3 | CTCFTYKDK ECVYYCHLDI I W | |

Note: X represents unrevealed amino acid present in Merck proprietary insulin variant.

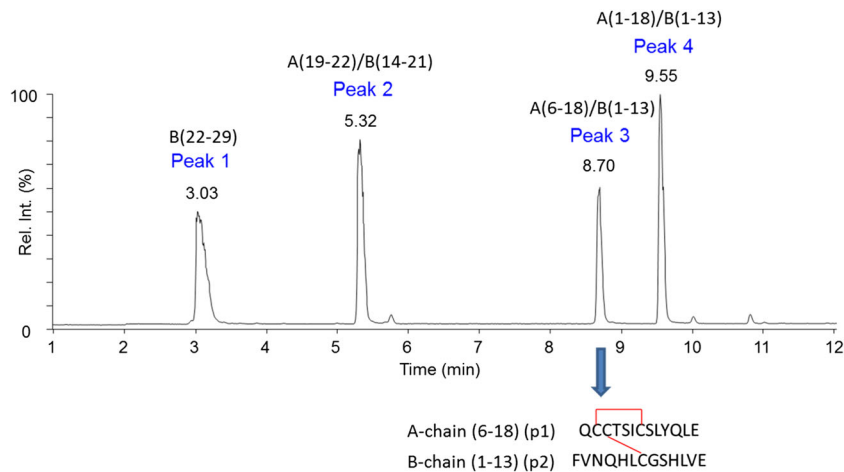


Figure 1. TIC of non-reduced peptide mapping of insulin variant following Glu-C digestion by reverse phase LC-MS. The amino acid sequence of peak 3 is shown under the peak with p1 and p2 indicated. “/” in peak designations represents the disulfide linkage

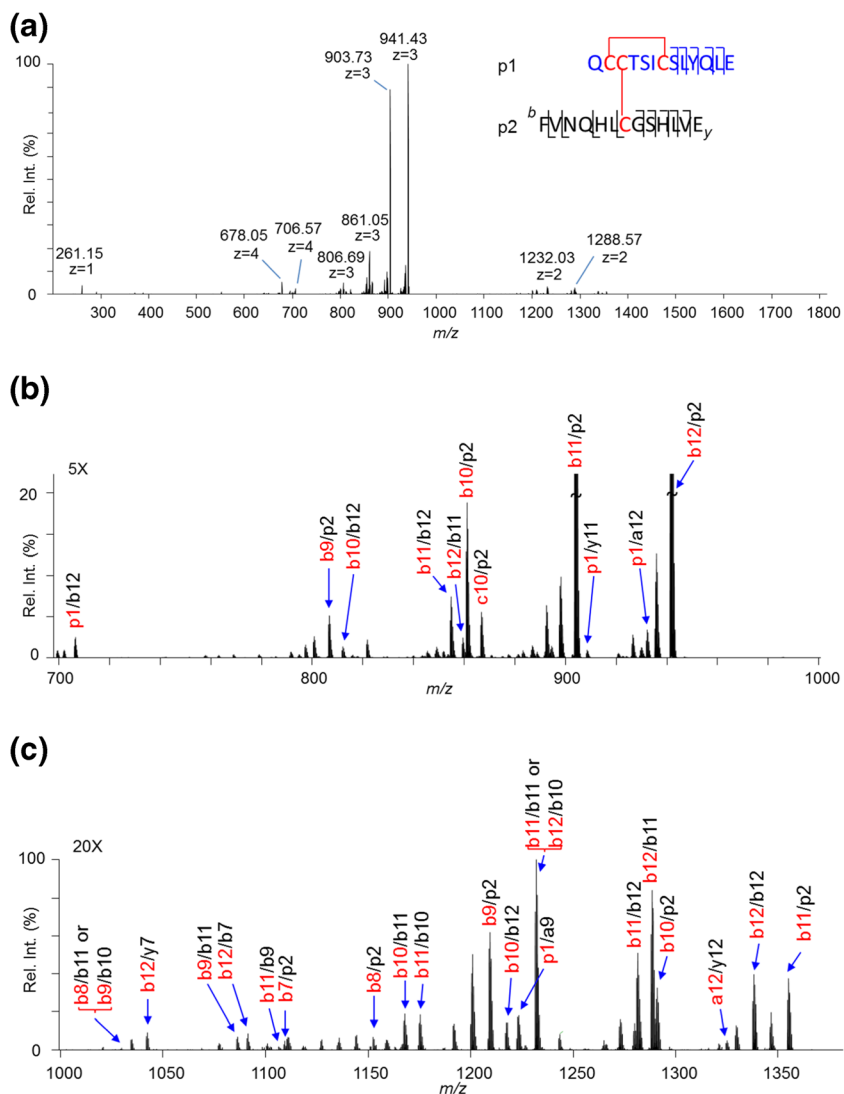


Figure 2. Mass spectra of insulin duplet peptide by CID MS/MS of precursor ion at m/z 743.08 (4+). (a) The MS/MS spectrum. (b) Zoomed in spectrum of 3+ charge state product ion region. (c) Zoomed in spectrum of 2+ charge state product ion region. The major b or y ions from p1 and p2 are labeled in red and black, respectively. The cleavage pattern of both p1 and p2 peptides is shown in (a)

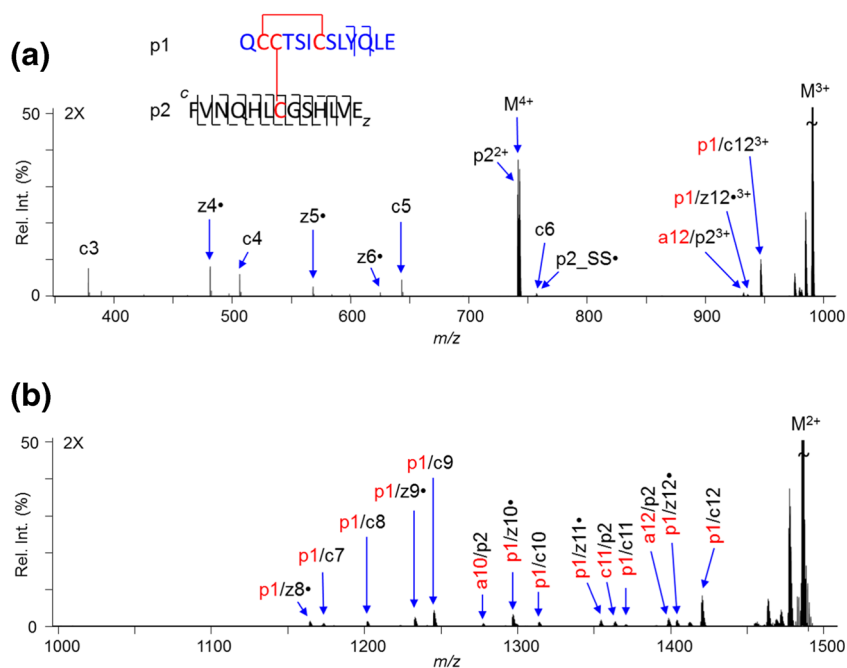


Figure 3. Mass spectra of insulin duplet peptide by ETD MS/MS of precursor ion at m/z 743.08 ($4+$). (a) Mass range of m/z 350–1000. (b) Mass range of m/z 1000–1500. Ions from p1 and p2 are labeled in red and black, respectively. The cleavage pattern of both p1 and p2 peptides is shown in (a)

developed to facilitate the assignment of peptide fragments. In our CID experiments, cleavage of disulfide bonds was not observed, although it was previously reported at a very low frequency [31, 44, 45]. The lack of fragmentation of the disulfide bonds limits the application of CID for mapping of disulfides.

ETD Fragmentation

The mass spectrum of targeted MS² by ETD for precursor ion m/z 743.08 ($4+$) of the model peptide is shown in Figure 3 and detailed information of the peaks are listed in supplemental Table S2. The backbone cleavage pattern with c and z ions is

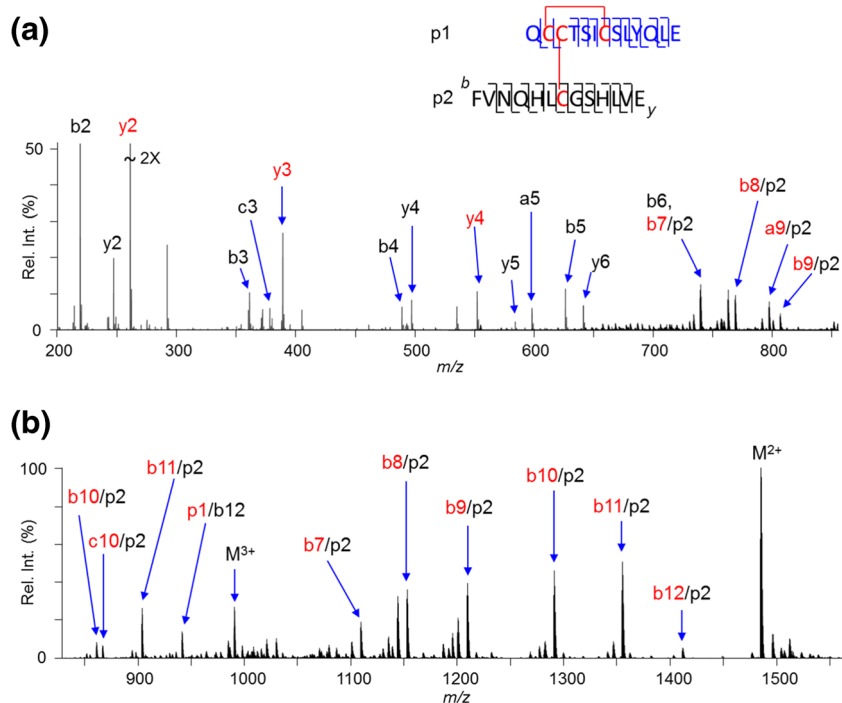


Figure 4. Mass spectra of insulin duplet peptide by ISD MS with major peaks labeled. (a) Mass range of m/z 200–850. (b) Mass range of m/z 800–1700. Ions from p1 and p2 are labeled in red and black, respectively. The cleavage pattern of both p1 and p2 peptides is shown in (a)

shown in inset of Figure 3a. In contrast to CID, most ions from ETD resulted from a single-backbone cleavage in p2 (Figure 3). However, p1 (p1-SS•) and p2 (p2-SS•) radical ions generated by the cleavage of the inter-chain disulfide bond were observed at an extremely low intensity (Table S2). Since there are 3 cysteine residues in p1, these ions are not sufficient to determine which cysteine residue in p1 is involved in the inter-chain disulfide linkage. Unfortunately, the intensities of the MS² fragment ions were too low for further MS³ characterization. Therefore, in this study, ETD did not preferentially cleave the disulfide bonds over the peptide bonds and was ineffective in cleaving intra-chain disulfide bonds, thus making it less useful in disulfide bond characterization.

ISD Fragmentation

The fragmentation mass spectrum of the insulin duplet peptide by ISD is shown in Figure 4. The backbone cleavage pattern with b and y ions is shown in the inset. The detailed peak list is shown in supplemental Table S3. All precursor ions at different charge states are fragmented by ISD, thereby generating much stronger signals of fragment ions than by CID and ETD methods.

Single-Backbone Cleavages and Intra-chain Disulfide-Bond Cleavage The major fragment ions generated by ISD were a series of b ions, (p1)_{b₇₋₁₂}/p2 in 2+ or 3+ charge states containing the intra-chain disulfide bond, due to a single-peptide backbone cleavage in p1 (Figure 4 and Table S3). Importantly, the simultaneous cleavages of both the intra-chain disulfide bond and the peptide backbone inside the disulfide-covered residues in p1 were also observed at a low intensity, resulting in a series of smaller fragment ions, (p1)_{b₄₋₆}/p2 and (p1)_{a₆}/p2 (Figure 5). These fragments still had the inter-chain disulfide bond intact but with a loss of p1-Cys7. Hence, either p1-Cys2 or p1-Cys3 forms the inter-chain disulfide bond with p2-Cys7, and p1-Cys7 is involved in the intra-chain disulfide with either p1-Cys2 or p1-Cys3. The cleavage of the disulfide bond in p1 was found to be symmetric between two S atoms: R₁-S-S-R₂ → R₁-SH + HS-R₂. Similar to ISD, the symmetric cleavage of a disulfide bond was reported using MALDI in-source decay [46]. In contrast to the disulfide fragmentation mechanism in CID proposed by others [45], the asymmetric cleavage of the disulfide bond was not observed in ISD experiments.

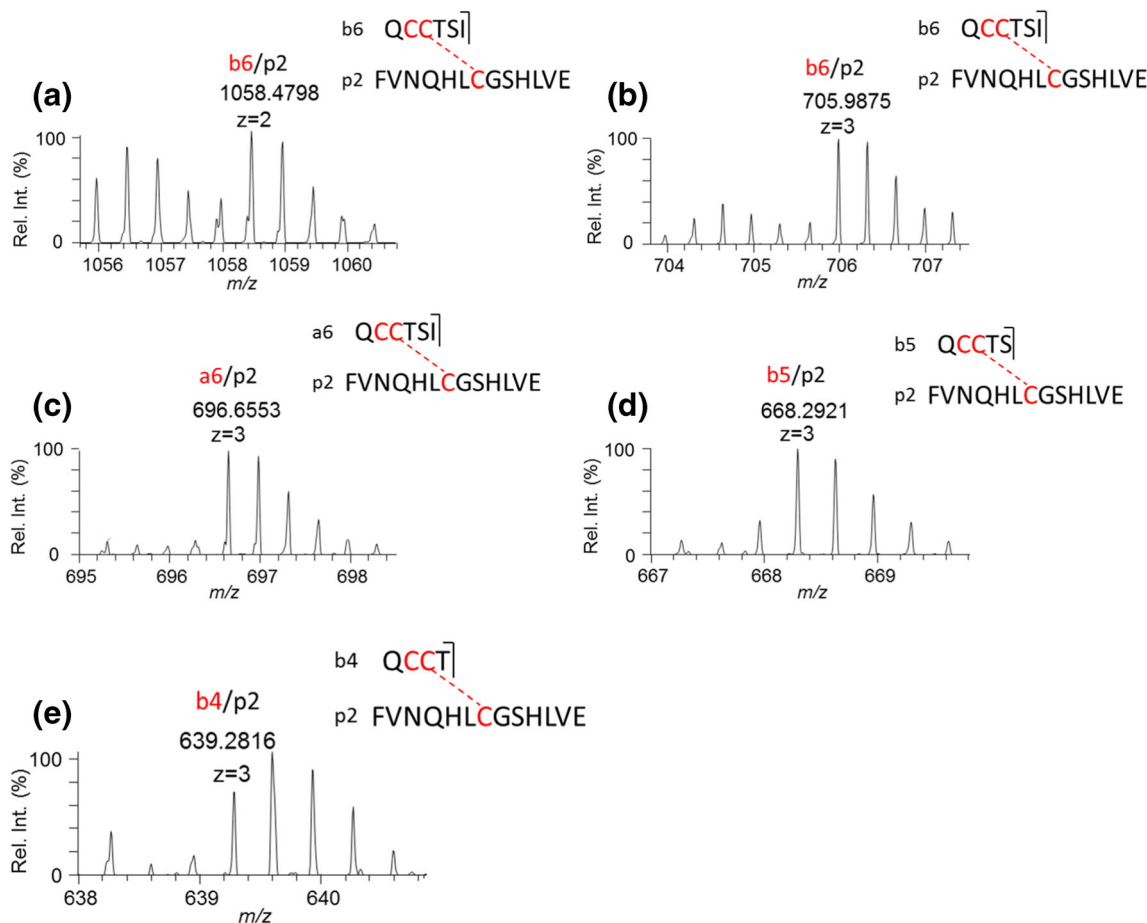


Figure 5. Mass spectra of insulin duplet peptide fragment ions from a single-backbone cleavage and intra-chain disulfide bond cleavage on p1 by ISD. (a) (p1)_{b₆}/p2 in 2+ charge state, (b) (p1)_{b₆}/p2 in 3+ charge state, (c) (p1)_{a₆}/p2 ion, (d) (p1)_{b₅}/p2 ion, and (e) (p1)_{b₄}/p2 ion. Ions from p1 and p2 are labeled in red and black, respectively. The peptide sequence and cleavage pattern is indicated for each ion

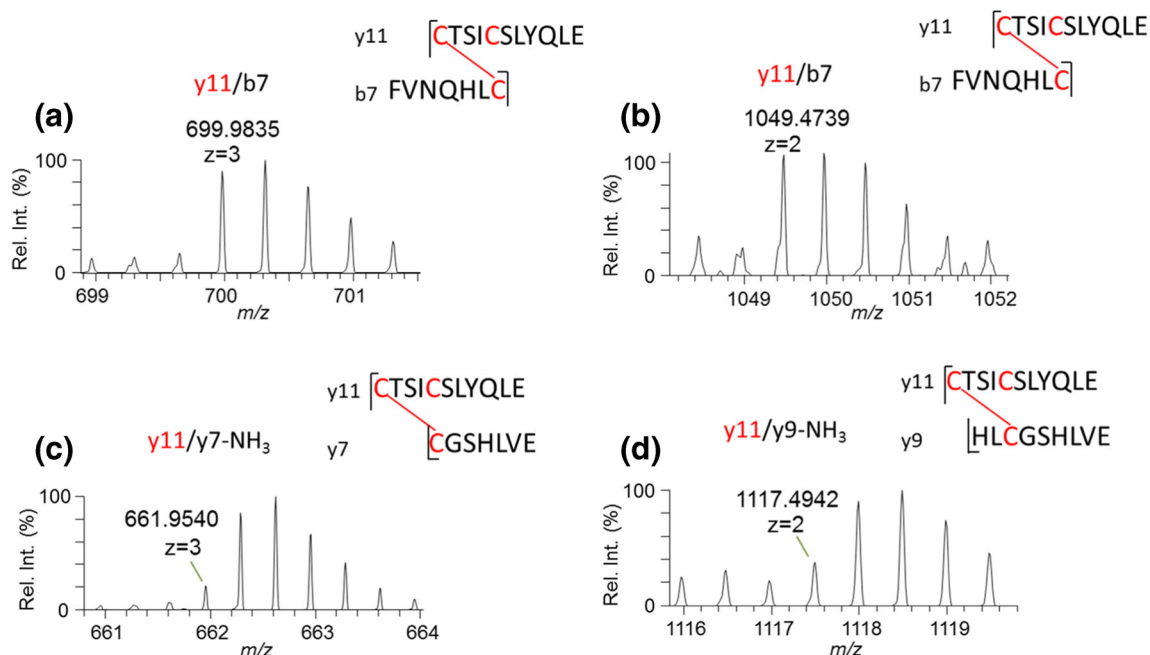


Figure 6. Mass spectra of insulin duplet peptide cleavage products from double-backbone cleavage and intra-chain disulfide bond cleavage by ISD. **(a)** $(p1)y_{11}/(p2)b_7$ in 3+ charge state, **(b)** $(p1)y_{11}/(p2)b_7$ in 2+ charge state, **(c)** $(p1)y_{11}/(p2)y_7$ -NH₃ ion, and **(d)** $(p1)y_{11}/(p2)y_9$ -NH₃ ion. Ions from p1 and p2 are labeled in red and black, respectively. The peptide sequence and cleavage pattern is indicated for each ion

Double-Backbone Cleavages and Intra-chain Disulfide-Bond Cleavage Many fragment ions (b/b ions) from the double-backbone cleavages of the duplet peptide by ISD were observed, including $(p1)b_7/(p2)b_{9-12}$, $(p1)b_8/(p2)b_{8,10,11,12}$, $(p1)b_9/(p2)b_{10-11}$, $(p1)b_{10}/(p2)b_{11-12}$, $(p1)b_{11}/(p2)b_{11-12}$, and $(p1)b_{12}/(p2)b_{10}$ (Table S3). The other types of double cleavage ions of the backbones including $(p1)a_7/(p2)b_{10-11}$, $(p1)a_8/(p2)b_{11}$, $(p1)a_9/(p2)b_{10-11}$, $(p1)a_9/(p2)y_7$, $(p1)b_9/(p2)y_7$ were also observed at a lower frequency. Like the single cleavage described above, cleavages in p1 peptide during these double cleavages occurred at residues after the intra-chain disulfide bond covered region, i.e. after p1-Cys7. These results suggest that residues outside the intra-chain disulfide bond covered region in p1 are more likely exposed to be cleaved during ISD. Most importantly, triple-cleavage ions, due to a double cleavage in p1/p2 and one intra-chain disulfide bond cleavage in p1 were clearly detected (Figure 6). These ions included $(p1)y_{11}/(p2)b_7$, $(p1)y_{11}/(p2)y_7$ -NH₃, and $(p1)y_{11}/(p2)y_9$ -NH₃ ions, and all had p1-Cys3, p1-Cys7 and p2-Cys7 present, with p1-Cys2 cleaved off. Since p1-Cys7 was involved in the

intra-chain disulfide linkage as described above, it can be unequivocally determined that p1-Cys3 forms the inter-chain disulfide bond with p2-Cys7 in these ions. In turn, p1-Cys2 can be assigned to form the intra-chain disulfide bond with p1-Cys7 as identified above (Figure 6).

Several new observations on peptide fragmentation can be made from our various dissociation experiments (Table 2). First, the double-backbone cleavage could occur at a relatively high frequency on the insulin duplet peptide by both ISD MS and CID MS/MS. Our results in this study expanded the current observation that only single cleavage is induced by CID MS/MS. Secondly, in contrast to CID MS/MS, ISD MS was also able to cleave disulfide bonds, generating various fragment ions through multiple modes of cleavages that allowed efficient and accurate assignments of disulfide bond linkages. Efficiency for cleavage of disulfide bond by ETD is very low, and thus MS² by ETD is not suitable for characterizing intra-chain disulfide linkage in our model peptide.

Table 2. Fragmentation Pattern and the Number of Detected Fragment Ions of Insulin Duplet Peptide (p1/p2) by CID, ETD, and ISD

| Dissociation method | Precursor ion charge state | Number of fragment ions | | | |
|---------------------|----------------------------|--------------------------|---|--------------------------|---|
| | | Single-backbone cleavage | Single-backbone cleavage and S-S cleavage | Double-backbone cleavage | Double-backbone cleavage and S-S cleavage |
| CID | 4+ | 16 | ND | 19 | ND |
| ETD | 4+ | 21 | ND | ND | ND |
| ISD | All | 23 | 3 | 16 | 3 |

Note: single S-S cleavage = intra-chain S-S cleavage. ND not detected

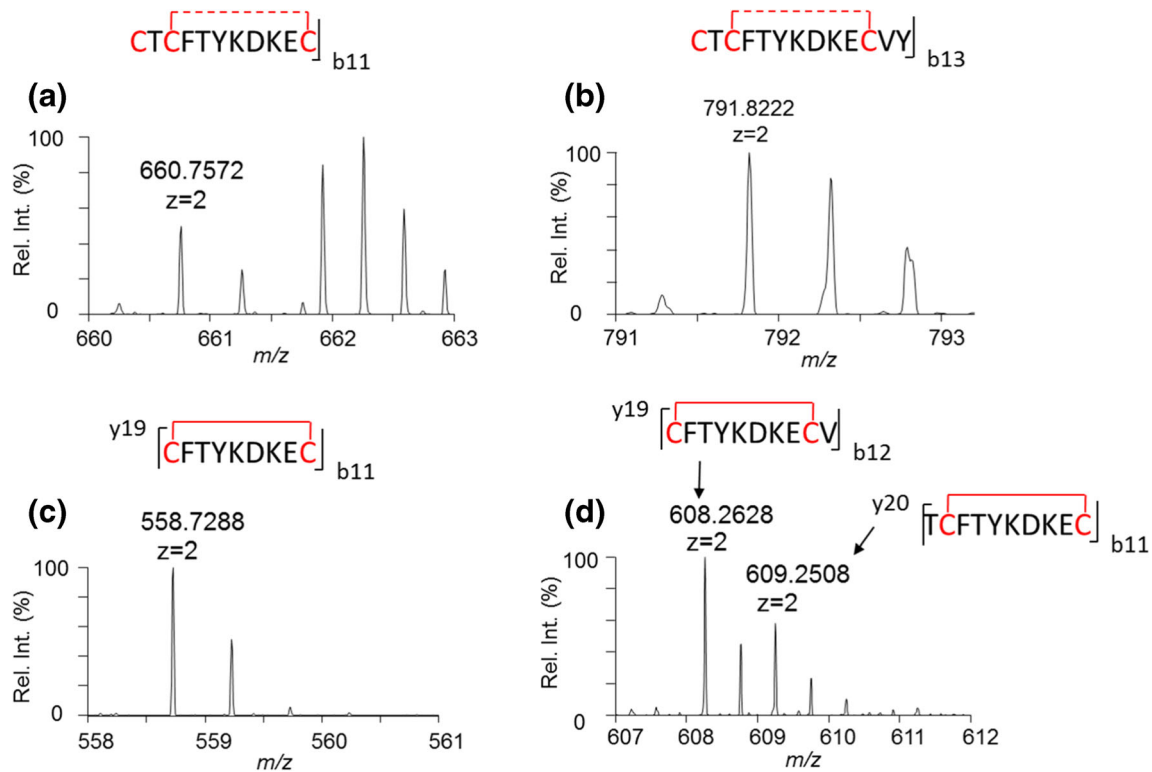


Figure 7. Mass spectra of endothelin 3 by ISD. Fragment ions from single-backbone cleavage and intra-chain disulfide bond cleavage, (a) b11 and (b) b13. Fragment ions from double-backbone cleavage, (c) y19-b11, and (d) y19-b12 and y20-b11

Characterization of Double Disulfide Bonds in Endothelin 3 and Relaxin 2 Peptides by ISD

As demonstrated above, ISD has been an efficient and successful method to determine disulfide linkages in the insulin duplet peptide. To determine whether the method is applicable to other disulfide containing proteins, endothelin 3 and relaxin 2 peptides were also characterized by ISD.

Endothelin 3 is a short peptide protein with the sequence of CTCFTYKDKECVYYCHLDIIW, and contains two nested disulfide bonds (Cys1-Cys15 and Cys3-Cys11). The endothelin 3 peptide was directly subjected to ISD. The fragment ions, b11 and b13, resulting from a single cleavage at backbone and a cleavage at one of disulfide bonds were

observed (Figure 7a, b). These fragments contained 3 cysteines, two of which formed a disulfide bond and the remaining cysteine had a free -SH group. Thus, the cysteine residues forming the disulfide linkages cannot be determined in b11 or b13 ions. Interestingly, the fragment ions, y19/b11, y19/b12, and y20/b11, from a double-backbone cleavage were detected (Figure 7c, d). All of these ions contained one disulfide bond, Cys3-Cys11. Therefore the other disulfide bond was readily determined as Cys1-Cys15.

Relaxin 2 is similar to insulin structurally in spite of the low sequence similarity. It consists of two peptide chains linked by three disulfide linkages (chain A: QLYSALANKCCHVGCTKRSLARFC, and chain B: DSWMEEVIKLCGRELVRAQIA

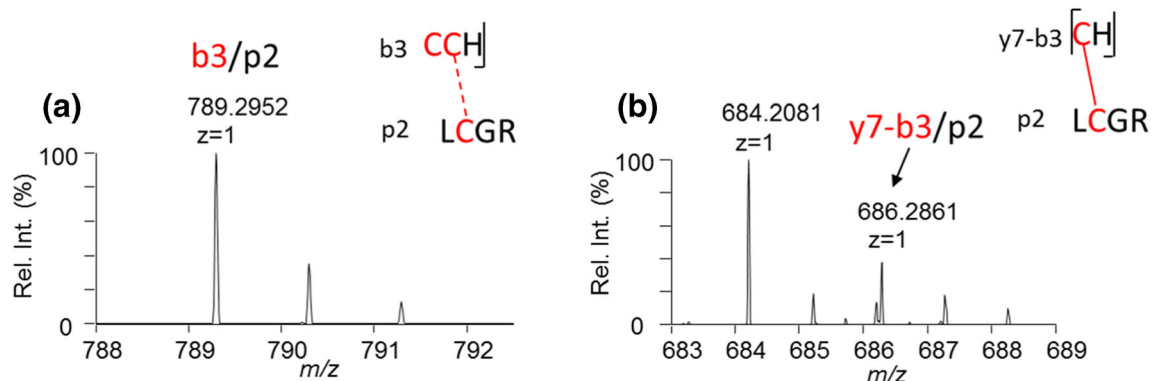


Figure 8. Mass spectra of relaxin 2 duplet peptide (p1)CCHVGCTK/(p2) LCGR from trypsin/LysC digestion by ISD. Fragment ions from single-backbone cleavage and intra-chain disulfide bond cleavage, (a) (p1)b3/p2. Ions from a double-backbone cleavage of the duplet peptide, (b) (p1)y7-b3/p2

ICGMSTWS). Following digestion by trypsin and Lys-C, relaxin 2 peptides were analyzed by LC-MS. The duplet peptide (p1)CCHVGCTK/(p2) LCGR contains an intra-chain disulfide bond in p1, and an inter-chain disulfide bond between p1 and p2. Following ISD, the smaller fragment ion, (p1)b3/p2, due to a backbone cleavage at b3 of p1 and the intra-chain disulfide cleavage, was detected (Figure 8a). This fragment ion contains p1-Cys1, p1-Cys2, and p2-Cys2. Therefore, which of two cysteines in p1 is involved in the inter-chain disulfide bond formation cannot be determined. Another fragment ion, (p1)y7-b3/p2, generated from a double-backbone cleavage from p1, was also clearly detected (Figure 8b). It contains p1-Cys2 and p2-Cys2. This unequivocally identified the inter-chain disulfide bond as p1-Cys2 connected to p2-Cys3 in the duplet peptide, and left the intra-chain disulfide bond as Cys1-Cys6 in p1.

The power of ISD in disulfide analysis using insulin, relaxin, and endothelin has been clearly demonstrated in this study. Direct characterization of disulfide bonds by ISD MS is simple and fast and can be readily performed in a mass spectrometer with a single-stage high-resolution mass analyzer for sequence and disulfide bond structure elucidation. This would expand the application of disulfide structure analysis by ISD MS in more laboratories. In addition, ISD MS eliminates the tedious experimental settings for the partial reduction method, and avoids potential disulfide scrambling issue that could occur during the partial reduction experiments.

Conclusion

Three gas-phase dissociation methods at either MS/MS level (CID and ETD) or MS level (ISD) were compared for disulfide linkage analysis of the insulin duplet peptide generated from Glu-C digestion under non-reducing condition. The cleavage patterns by these methods are significantly different. The CID MS/MS cleavages occur mostly on the p1 peptide from chain A and much less on p2 from chain B. In contrast, ETD MS/MS mostly induces single cleavages in p2 and is able to only cleave the inter-chain disulfide at a low level, and thereby is not suitable for analysis of the intra-chain disulfide linkages in this model peptide. ISD MS is the only gas-phase fragmentation method that is able to induce simultaneous cleavages in both peptide backbone (single or double cleavages) and disulfide bond (Table 2), providing useful fragment ions to assign both inter-chain and intra-chain disulfide linkages. The usage of ISD is also clearly demonstrated in several other disulfide containing proteins including relaxin and endothelin.

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