Characterization of Disulfide Linkages in Peptides Using Tandem Mass Spectrometry (MS^n)

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OVERVIEW

- Disulfide regio-isomers of peptides containing four cysteine residues (two disulfide bonds), were synthesized and then subjected to tandem mass spectrometry (MS^n).
- The objective was to use internal fragment ions to identify disulfide bond connecting patterns and understand the fragmentation chemistry of these isomers.
- It was observed that the internal fragment ions also showed very similar fragmentation patterns.
- The possible mechanism contributing to these similar fragmentation patterns were investigated.
- Results indicated that disulfide bonds can undergo consecutive C-S bond cleavages yielding b and y ions of identical *m/z*.

INTRODUCTION

- Disulfide bond formation is one of the most common post translational modifications to occur in proteins. Deciphering the structures and biological functions of proteins in living organisms is a vital tool for understanding cellular processes [1] .
- Collision-induced dissociation (CID) typically provides sequence information from the exocyclic region of peptides which contain a disulfide bond. This imposes difficulties in the structural characterization of the region constrained by the disulfide loop.[2]
- Tandem mass spectrometry (MS^n) on ions formed from internal loss of several amino acids (AA) has been used to identify the absolute configuration of disulfide bonds in peptides containing multiple disulfide bonds [3,4] .

METHODS

MASS SPECTROMETRY

- Nanoelectrospray ionization (NanoESI) was used for formation of peptide ion.
- All CID data were collected on a hybrid triple quadrupole-linear ion trap (AB SCIEX 4000Qtrap) mass spectrometer.
- Thermolysin digestion was used as an orthogonal method for disulfide bond linkage determination.

SEPRARATION

- Agilent Technologies 1200 series HPLC for peptide isomer separation.
- Gradient 20- 35% **B** in 30 minutes.
  - **A** composition is 0.1% TFA/H_2O
  - **B** composition is 80% ACN/20% H_2O/0.085% TFA
- Flow rate: 0.65 mL/min.
- Eclipse XDB-C18 column was used for separation.

MATERIALS

- Peptides were prepared to a final concentration of 10 μM in 50:50 (v:v) MeOH: H_2O solutions with 1% HOAc added for positive ion mode nanoESI.
- Oxidized peptides were obtained by reacting excess [Pt en$_2$ (OH)$_2$Cl] with fully reduced peptides (Pt(IV): peptide = 3:1, molar ratio) in 50:50 MeOH:H_2O at room temperature (RT) for 3-5 hours.
- Sep-Pak C18 1cc column was used for desalting.
- Peptic digest were obtained by reacting thermolysin with the oxidized peptide in a 10:1 (peptide: thermolsyin) ratio in a 100mM Tris-HCl buffer (pH 6.5) at 65-70ºC for 30 minutes.

TABLE 1. PEPTIDES CONTAING TWO INTRACHAIN DISULFIDE BONDS

Peptide	Structure	NAME
P1 CARICAKLCLEVCK	CARICAKLCLEVCK	P1-I
	CARICAKLCLEVCK	P1-II
	CARICAKLCLEVCK	P1-III
P2 CAEKCIEKCLVRC	CAEKCIEKCLVRC	P2-I
	CAEKCIEKCLVRC	P2-II

RESULTS and DISCUSSION

FORMATION OF S-S BONDS

CARICAKLCLEVCK (MW : 1551.7Da) → [Pt en$_2$ (OH)$_2$ Cl], 3-5 hours @ RT → P1-I, P1-II, and P-III (MW : 1547.7Da)

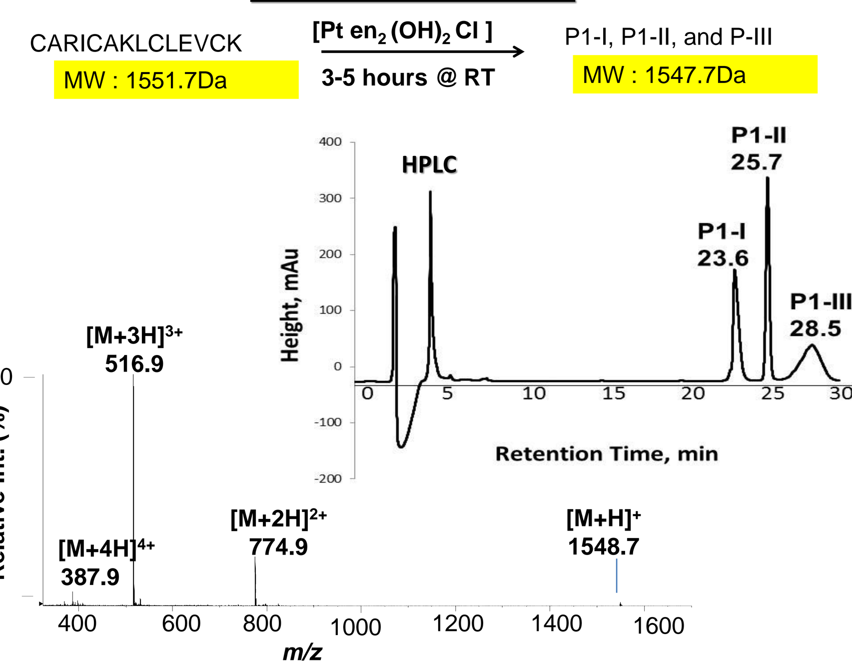


Figure 1. Synthesis, MS, and HPLC separation of the P1 peptide. MS revealed the formation of 2 S-S bonds and HPLC successfully separated the isomers.

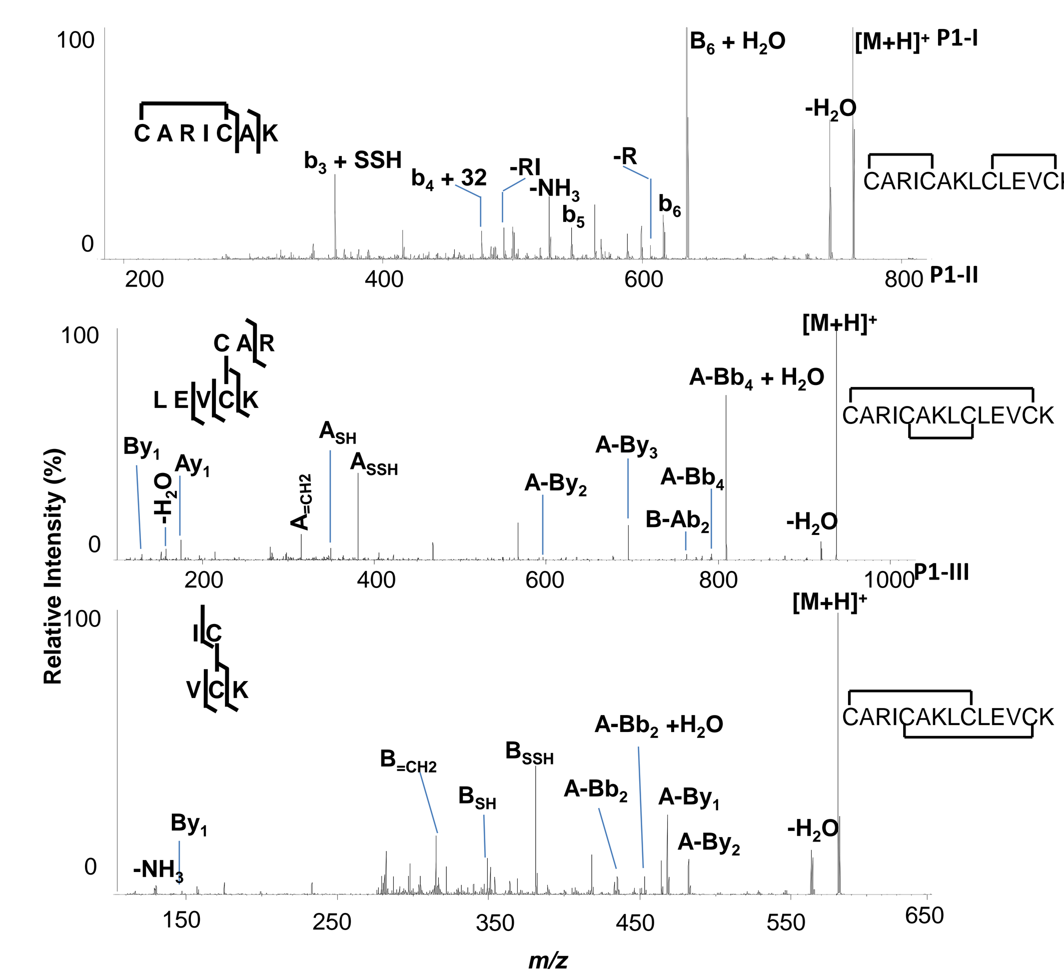


Figure 2. MS^2 of thermolysin digest fragments of the P1 peptide showing correct disulfide bond connecting patterns.

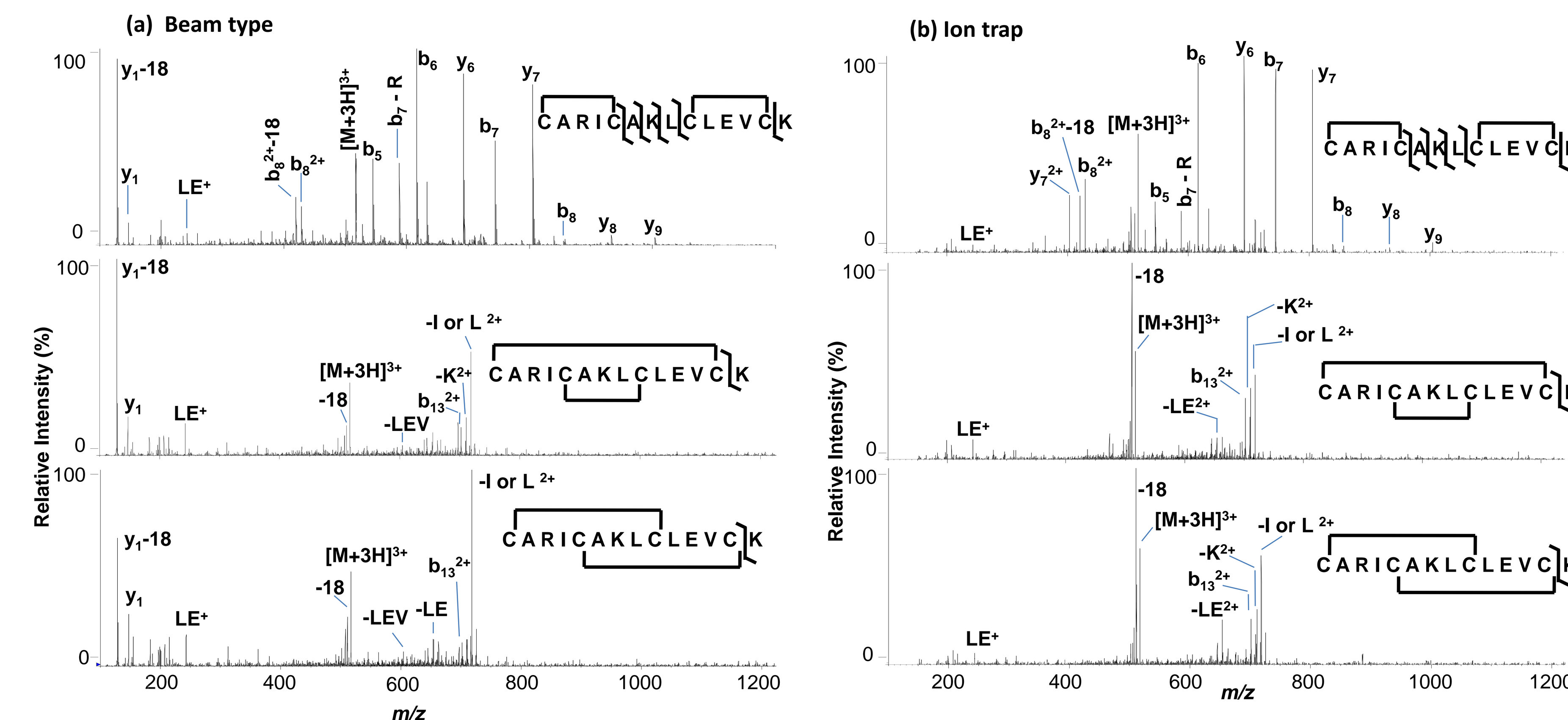


Figure 3. MS^2 of the triply charged P1 peptide. Both spectra clearly distinguish isomer P1-I. However, P1-II and P1-III where the disulfide bonds cover the entire sequence show limited back backbone fragments and yield identical spectra.

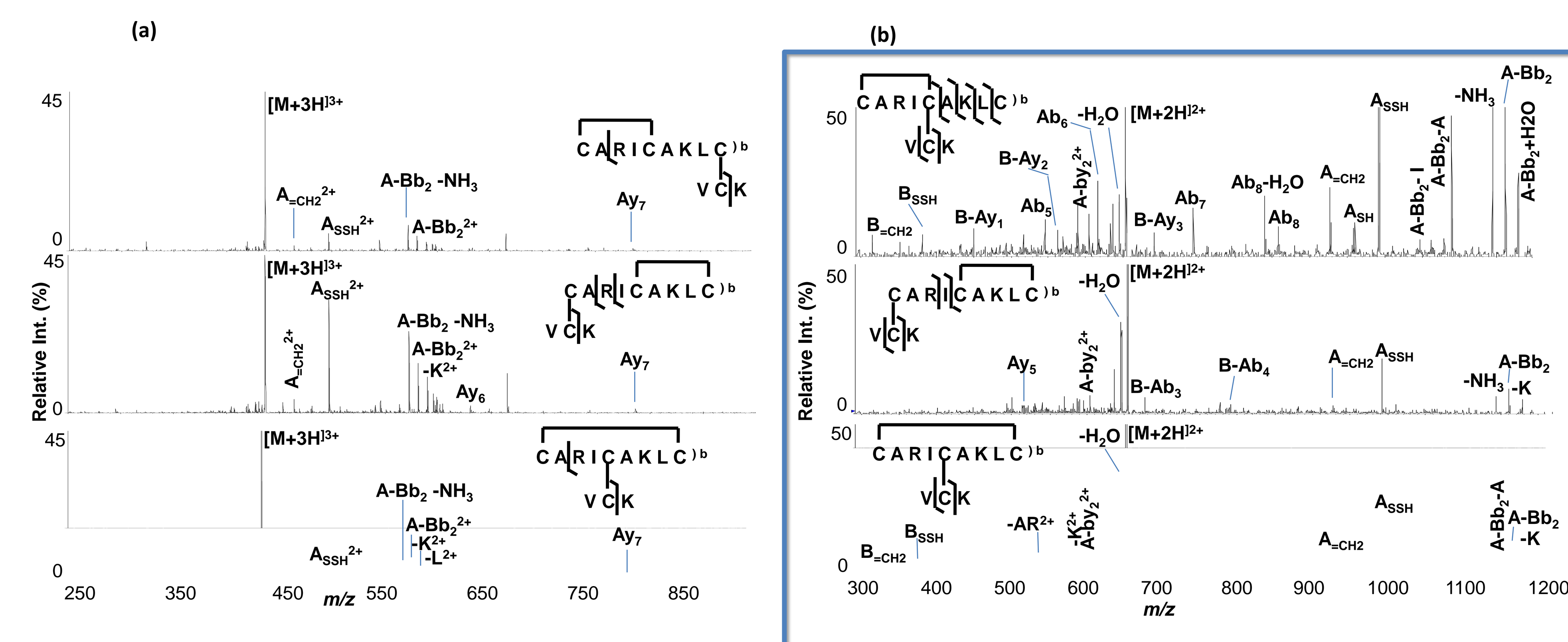
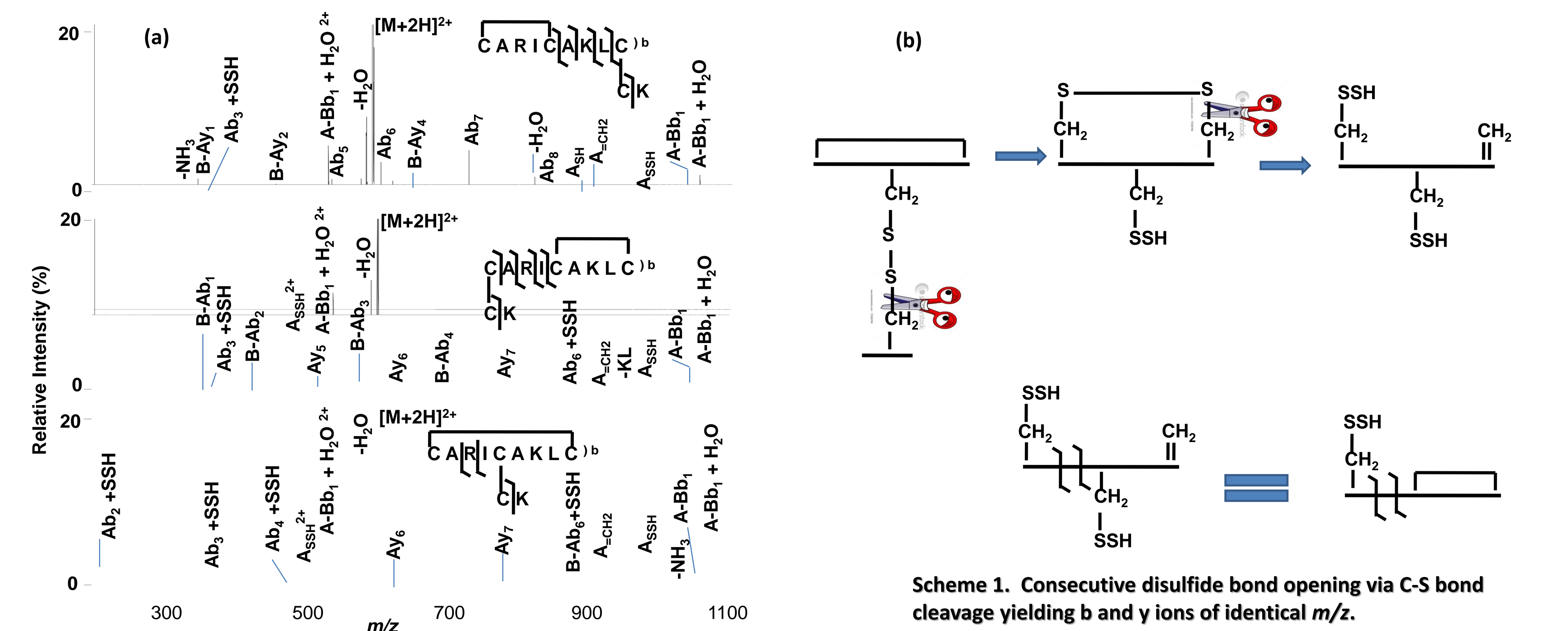


Figure 4. **(a)** MS^3 of the triply charged internal loss of LE show similar fragmentation. Contrary to the fact that the CID conditions are identical, isomer P1-II has a higher relative intensity of all major peaks observed. **(b)** MS^3 of the doubly charged however, fragmented exactly as expected showing a DISTINCTIVE fragmentation pattern for each isomer.



Scheme 1. Consecutive disulfide bond opening via C-S bond cleavage yielding b and y ions of identical *m/z*.

Figure 5. **(a)** MS^3 of the doubly charged internal loss of LEV showed no distinguishable fragmentation between isomers II and III. A hypothesis was proposed to explain the similar peaks observed **(b)**. In P1-III the observation of several b+ SSH peaks would support the proposed hypothesis.

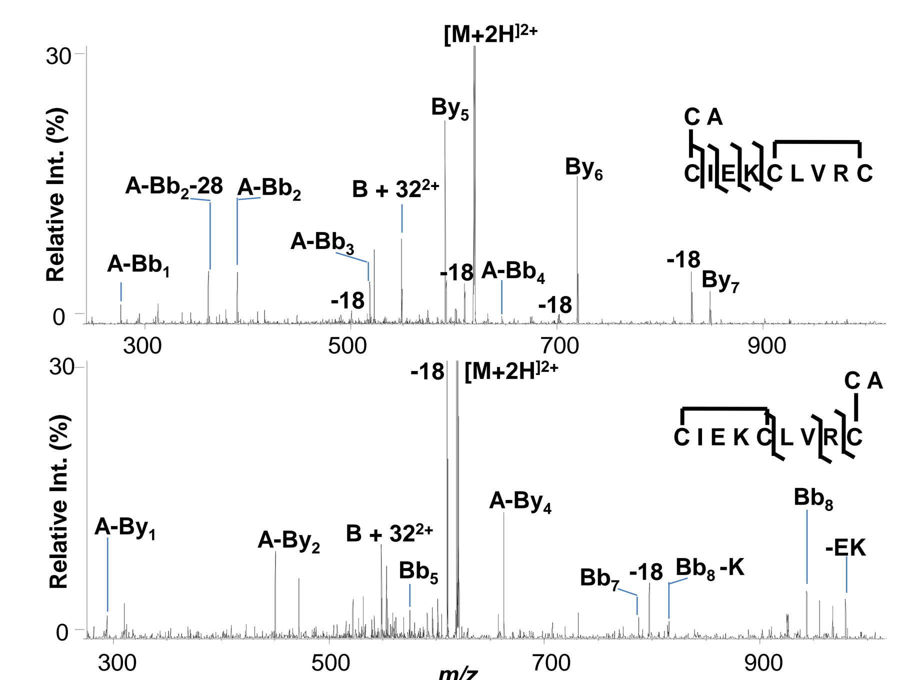


Figure 6. MS^3 of the doubly charged loss of EK from the P2 peptides. Unique fragmentation was observed for each isomer.

CONCLUSION

- With careful observation and familiarity, MS^3 of internal fragment ions can be used to decipher disulfide bond connecting patterns.
- In some cases, consecutive disulfide bond opening via C-S bond cleavage may lead to a series of similar b and y ions.
- To the best of our knowledge, this is the first time that three isomers containing two intrachain disulfide bonds were synthesized and their fragmentation patterns compared side by side.
- It should be noted, that the P2 peptide was commercially bought, but only two isomers could be successfully synthesized, therefore we do not know what the fragmentation chemistry of the third isomer would have revealed.

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