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Formation of c- and z-ions due to preferential cleavage at the N—C bond of Xxx-Asp/Asn residues in negative-ion CID of peptides

Nami Sugasawa[a], Taiji Kawase[b], Motoji Oshikata[b], Ryunosuke Iimuro[a], Akira Motoyama[c], Mitsuo Takayama[a,*]

[a] Yokohama City University, Mass Spectrometry Laboratory, 22-2 Seto, Kanazawa-ku, Yokohama, Japan
[b] Nihon Waters, Technology Application Department, Nihon Waters K.K., 1-3-12 Kitashinagawa, Shinagawa, Tokyo, Japan
[c] Shiseido, Analytical Research Group, 2-2-1 Hayabuchi, Tsuzuki-ku, Yokohama, Japan

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ABSTRACT

In collision-induced dissociation (CID) experiments of peptides, the formation of c- and z-ions originating from N—Cα bond cleavage of the peptide backbone is unusual, whereas b- and y-ions are often observed in positive-ion CID spectra. Here we report that characteristic product c- and z-ions can be observed in negative-ion CID spectra of peptides with Asp and Asn residues. The negative c- and z-ions are mainly generated at the N—Cα bond of Xxx-Asp and Xxx-Asn residues that are classified as the flexible amino acids in protein chemistry. In the absence of any acidic amino acid residues, negative-ion CID spectra show dominant c-ion peaks originating from cleavage at the N—Cα bond lying in between the 3rd and 4th residues from the C-terminus of analyte peptides. It is proposed that the N—Cα bond cleavage leading to the formation of c- and/or z-ions is caused by the negative charge located on the amide nitrogen, which generated with an intra-molecular hydrogen abstraction from the backbone amide regions due to negative-charged acidic functional groups.

1. Introduction

Tandem mass spectrometry (MS/MS or MS^n) coupled to electrospray ionization (ESI) [1,2] is a powerful analytical tool for analyzing organic and biological analytes. There are many studies that analyze collision-induced dissociation (CID) spectra of protonated peptides [3,4] and deprotonated peptides [5]. In particular, the mechanism(s) for the formation of b- and y-ions, originating from cleavage at the amide bond C—N of the peptide backbone, have been explained by the concepts of mobile proton [6] and acid-initiated selective cleavage at Asp/Glu/Cys-Xxx residues [7–10] in protonated peptides $[M+nH]^{n+}$. In the case of deprotonated peptides $[M-nH]^{n-}$, the product ion spectra can be characterized as follows:

a) The loss of neutrals such as H_2O, NH_3, CH_3COH (of Thr) and CHOH (of Ser) from b-, y- and precursor ions [10,11].
b) The selective C—N bond cleavage of Xxx-Asp/Glu/Cys residues [10,12].
c) The formation of c-ions originating from cleavage at N—Cα bonds of the backbone [12,13].

The formation of c-ions in mass spectrometric analysis of peptides and proteins is well known in in-source decay (ISD) [14,15] in matrix-assisted laser desorption/ionization (MALDI) MS and electron capture dissociation (ECD) [16] and electron transfer dissociation (ETD) [17] of ESI MS. In the CID study of peptides, however, the identification of c-ions has only been described in negative-ion mode [12,13]. Pu et al. of Cassady's group have reported that when using an alanine heptamer AAAAAAA and its single arginine (R) or lysine (K) containing analogs, the CID spectra of deprotonated analytes gave a dominant c4 and minor c_n-ions (n = 2, 3, 5, 6) independent of the position of the R and K residues [18]. Bokatzian-Johnson et al. of the same group recently reported definite mechanisms for c-ion formation of deprotonated peptides, i.e., the backbone N-Cα bond cleavage with the carboxyl (C)-terminal side 6- or 9-membered ring formation [13].

In the present paper, the formation of c- and z-ions in low-energy CID experiments with deprotonated peptides $[M-nH]^{n-}$ is described using ESI MS. The resulting amino (N)-terminal side c-ions are accompanied with the formation of C-terminal side z-ions

* Corresponding author. Tel.: +81 45 787 2431.
E-mail addresses: n135213b@yokohama-cu.ac.jp (N. Sugasawa), Taiji_Kawase@waters.com (T. Kawase), Motoji_Oshikata@waters.com (M. Oshikata), n135202e@yokohama-cu.ac.jp (R. Iimuro), akira_motoyama@jahoo.co.jp (A. Motoyama), takayama@yokohama-cu.ac.jp (M. Takayama).

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Table 1
Sequence and monoisotopic mass (M_m) of analyte peptides.

Peptide	Sequence (M_m)
ACTH19-36	PVKVYPNGAEDESAEAFP (1918.9)
[Arg^{22}]-ACTH22-39	RYPNGAEDESAEAFPLEF (2040.91)
[Arg^{36}]-ACTH19-36	PVKVYPNGAEDESAEAFR (1977.95)
[Arg^{36}]-ACTH18-36	RPVKVYPNGAEDESAEAFR (2134.05)
RLG	RLGNQWAVGDLAE (1427.71)
K5	KKKKK (658.48)

in terms of specific cleavage at the N—Cα bond of Xxx-Asp and Xxx-Asn residues of analyte peptides.

2. Experimental

2.1. Materials

All peptide samples were purchased from the Peptide Institute (Minoh, Osaka, Japan). The samples used are adrenocorticotropic hormone (ACTH) analog peptides, a model peptide (RLG) and a lysine pentamer (K5). The sequences and mono-isotopic masses (M_m) of all peptides are summarized in Table 1. Acetic acid and acetonitrile (HPLC grade) were purchased from Wako Pure Chemicals (Osaka, Japan). Water used in all the experiments was purified with a MilliQ water purification system from Millipore (Billerica, MA, USA).

2.2. Mass spectrometry

The ESI and CID experiments were performed on a SYNAPT G2 HDMS quadrupole time-of-flight mass spectrometer equipped with an ESI source and a MassLynx data processor (Waters Corp., Milford, MA, USA), an HCT ultra ETD II ion trap mass spectrometer (Bruker Daltonics Inc., Bremen, Germany), and a 6530qTOF mass spectrometer (Agilent Inc., Santa Clara, CA, USA). The sample was introduced into the ion source at a flow rate of 3–300 μL/min with nitrogen being used as both nebulizing and drying gas.

2.3. Sample preparation

The samples were prepared as 10 mM solutions with a 1:1 (v/v) mixture of acetonitrile/water. 1–2.5 mL aliquots of 10 mM solutions of each peptide in water/acetonitrile (1:1, v/v) were injected into a flow-injection LC/MS system equipped with an electrospray ion source. The samples were 1–10 μM solutions with a 50:50:1 mixture of acetonitrile/water/acetic acid as the ESI solvent.

3. Results and discussion

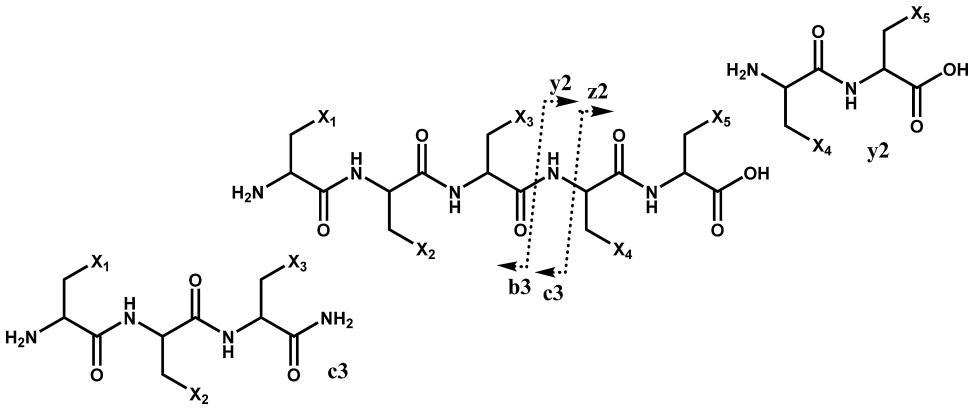
3.1. Preferential cleavage at the N—Cα bond of Xxx-Asp and Xxx-Asn residues to form c- and z-ions

Fig. 1 shows the negative-ion CID spectra of doubly-deprotonated analytes $[M-2H]^{2-}$ of ACTH analogs. The striking features of these CID spectra are the appearance of N-terminal side c-ions and C-terminal side z-ions, i.e., c10, c6, z8 and z12 (Fig. 1a), c7, c3, z11 and z15 (Fig. 1b), c10, c6, z8 and z12 (Fig. 1c), and c11, c7, z8 and z12 (Fig. 1d), originating from preferential cleavage at the N—Cα bond of Glu-Asp (E-D) and Pro-Asn (P-N) residues. An internal fragment ion NGAE after loss of H_2O was also observed. According to the nomenclature of peptide fragments [19], the C-terminal side z-ions are corresponding to the loss of NH_3 from y-ions (Scheme 1). As expected in negative-ion CID experiments, precursor ion $[M-2H]^{2-}$, y- and z-ions were observed with the loss of neutral H_2O, while the resulting c-ions were not accompanied by a noticeable loss of neutrals. Furthermore, the C-terminal amino acid residues corresponding to y1 ions were observed in the lower m/z region, i.e., proline (P) (Fig. 1a), phenylalanine (F) (Fig. 1b), arginine (R) (Fig. 1c), and R (Fig. 1d). Furthermore, it has already been reported that the observed negative y-ions originate from cleavage at the C—N bond at the N-terminal side of acidic amino acid residues Xxx-Glu and Xxx-Asp [10]. It should be noted here that c-ions were observed accompanying with z-ions originating from the cleavage at the N—Cα bond of Glu-Asp and Pro-Asn residues.

In order to confirm the selective cleavage at the N—Cα bond of E-D and P-N residues described above, the negative-ion CID spectrum of $[M-2H]^{2-}$ for another peptide, namely RLG which contains D and N residues was obtained as shown in Fig. 2. The CID spectrum shows the striking peak of c-ions (c3 and c9) and z-ions (z4 and z10) originating from cleavage at the N—Cα bond of G-N and G-D residues, suggesting a selective cleavage at the N—Cα bond of Xxx-Asp and Xxx-Asn residues. Furthermore, the C-terminal y1 ion (E), internal ions and z-related ions such as z_n - E (n = 4, 10) and doubly-charged z-ion $[z10]^{2-}$ were observed. From the negative-ion CID experiments with ACTH analogs and RLG, it can be concluded that preferential cleavage at the N—Cα bond of Xxx-Asp and Xxx-Asn residues occurs to form c/z-ion pair. The formation of y1 ions observed in all the CID spectra can be rationalized by a mechanism of "negative charge-initiated selective cleavage" at the C—N bond of Xxx-Asp/Glu residues [10], as illustrated in Scheme 2.

3.2. Mechanism of c-ion formation

Harrison and Young first reported a possible structure of negative c-ions (Scheme 3) [12]. With respect to the formation of c-ions



Scheme 1. The nomenclature of peptide fragments b, y, z and c.

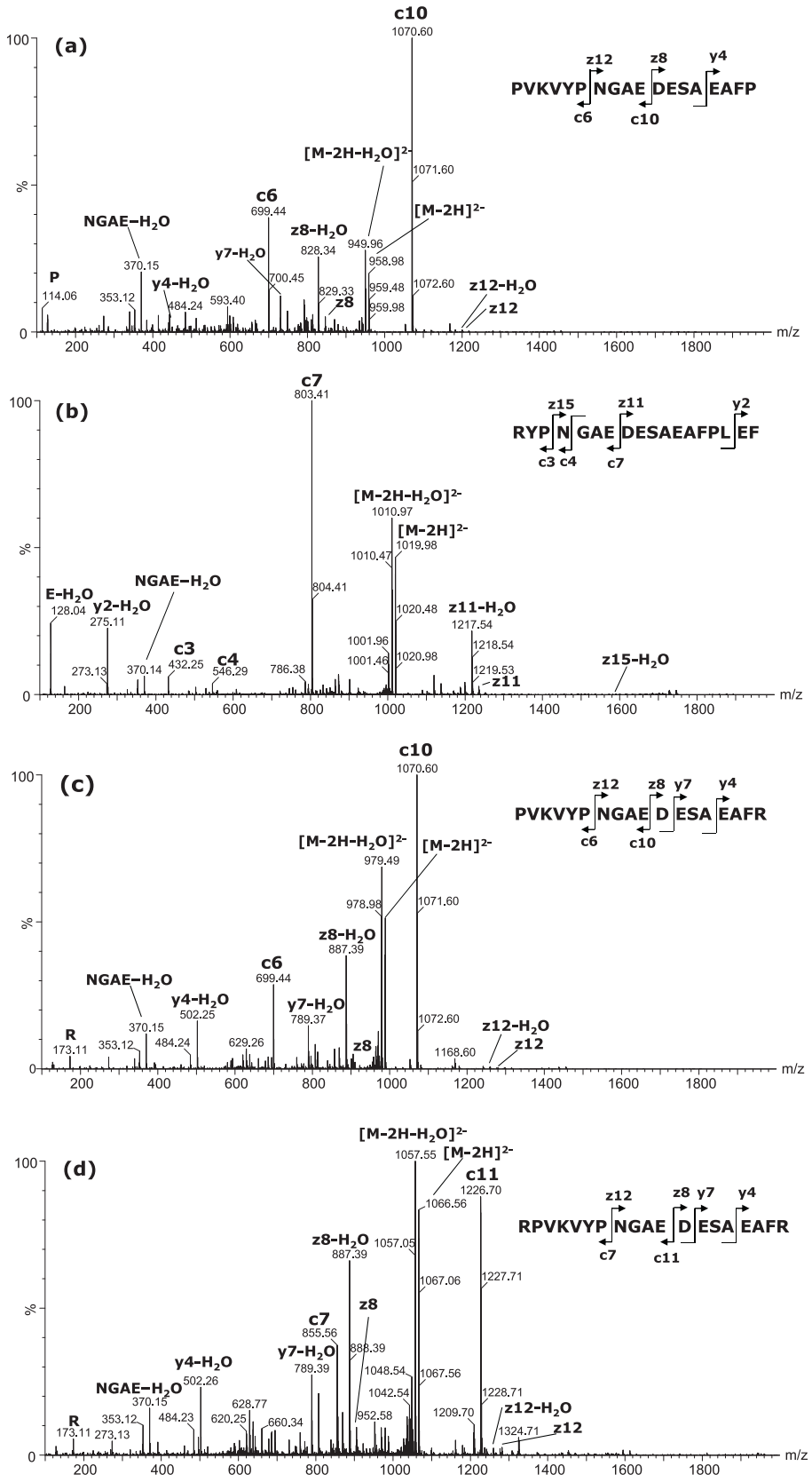


Fig. 1. Negative-ion CID spectra of doubly-charged analytes $[M-2H]^{2-}$ for ACTH analog peptides: (a) ACTH19-36 (M_m 1918.9), (b) [Arg^{22}]-ACTH22-39 (M_m 2040.91), (c) [Arg^{36}]-ACTH19-36 (M_m 1977.95) and (d) [Arg^{36}]-ACTH18-36 (M_m 2134.05).

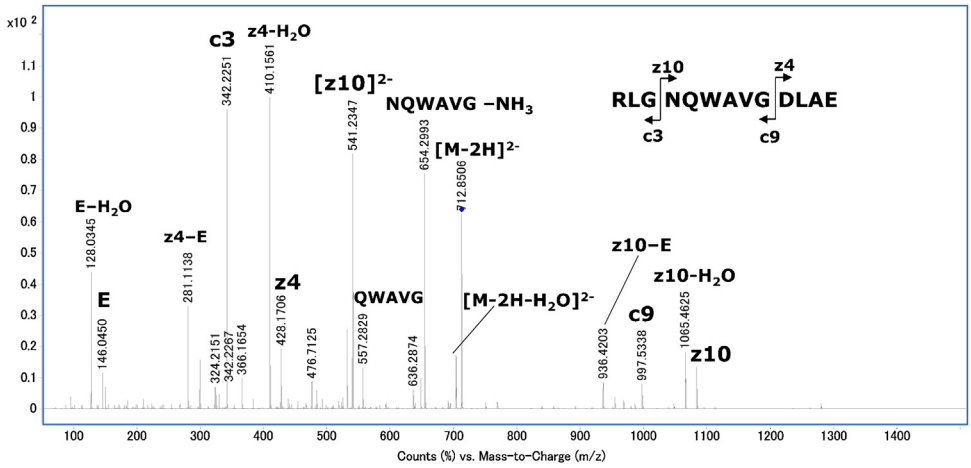
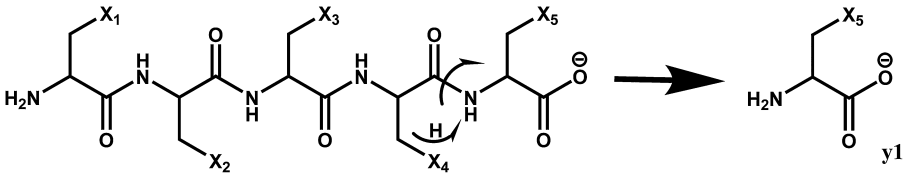
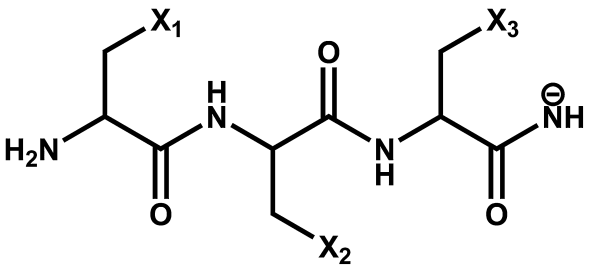


Fig. 2. Negative-ion CID spectrum of doubly-charged analytes $[M-2H]^{2-}$ for the RLG peptide (M_m 1427.71).



Scheme 2. Mechanism for y1 ion formation in negative-ion CID of peptides.

in negative-ion CID of peptides, the most informative data have been reported by Pu et al. [18]. They have shown that negative-ion CID spectra of alanine heptamer analogs AAAAAAA (A7), RAAAAAA, AAARAAA, AAAAARA, AAAAAAR, KAAAAAA, AAAKAAA, AAAAAKA and AAAAAAK give dominant c4 ions and minor c_n ions (n = 1, 2, 3, 5, 6, 7), with the exception of RAAAAAA. The CID spectra of A7 analogs suggest a site specific cleavage at the N—Cα bond lying in between the 3rd and 4th residues from the C-terminus. Furthermore, Bokatzian-Johnson et al. recently reported a comprehensive study of the formation of c-ions in the CID experiments of deprotonated peptides with C-terminal amide and acid groups [13]. They have found a tendency that in negative-ion CID of the analytes without any acidic amino acid residues the C-terminal amide peptides gave c_{m-2} ions originating from cleavage at the N—Cα bond lying in between the 2nd and 3rd residues from the C-terminus, while the C-terminal acid peptides gave c_{m-3} ions cleaved at the N—Cα bond between the 3rd and 4th residues. On the basis of the results obtained there, they have proposed the mechanism of C-terminal side 6- and 9-membered ring formation for the formation of c_{m-2} and c_{m-3} ions, respectively. And also, they reported that both negative-ion CID spectra of cholecystokinins (DYMGWMDF-NH_2 and -OH) gave c_{m-3} ion corresponding to c6 originating from the cleavage at the N—Cα bond of Xxx-Asp residue, coinciding with our result obtained here.



Scheme 3. Structure of negative c-ion proposed by Harrison and Young [12].

In order to confirm the mechanism of C-terminal side 9-membered ring formation by Bokatzian-Johnson et al., here we performed CID experiments of deprotonated analyte $[M-H]^-$ of lysine pentamer K5 (Fig. 3). The CID spectrum shows abundant c2 and minor c3 product ions. The major product c2 ion is corresponding to c_{m-3} ion assigned by Bokatzian-Johnson et al. [13]. The peptide K5 does not contain any acidic amino acid residues. Thus, the negative charge of the precursor ions $[M-H]^-$ generated immediately after the evaporation from charged droplets in the electrospray processes should be located on the C-terminal carboxyl group. In the case of C-terminal acid peptides without any acidic amino acid residues, the formation of negative c-ions has to be explained by the mechanism of 9-membered ring formation, as shown in Scheme 4. In Scheme 4, the negatively charged C-terminal carboxylate may abstract a proton from the backbone amide hydrogen between the X2 and X3 residues before the cleavage at the N—Cα bond, suggesting that the N—Cα bond cleavage is caused by the negative charge located on the amide nitrogen.

3.3. Mechanistic considerations of c/z-ion pair formation

In the case of peptides with Asp and Asn residues, the CID experiments resulted in significant negative c- and z-ions originating from preferential cleavage at the N—Cα bond of Xxx-Asp/Asn residues independent of the sites of those residues (Figs. 1 and 2). Although preferential cleavage at the N—Cα bond of Xxx-Asp/Asn residues to form c-ions has been reported in the analysis of flexibility of intact proteins [20], the residue specific c/z-ion pair formation in the CID experiments has not been reported so far. It has been reported that in negative-ion CID experiments of peptides, the selective cleavage of the backbone C—N or N—Cα bonds of acidic residues such as Xxx-Asp/Glu/Cys takes place to form b-, y- and/or c-ions [10]. However, the cleavage at the N—Cα bond of Xxx-Glu residues does not occur to form c- and z-ions, though the Glu residue is very similar to the Asp residue in structure and acidic nature. A difference in length of methylene chain between Asp and Glu residues may explain the preferential cleavage at the N—Cα bond of Xxx-Asp/Asn residues,

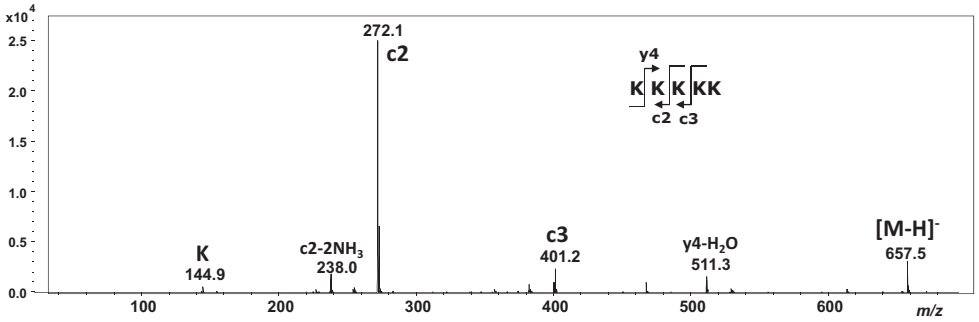
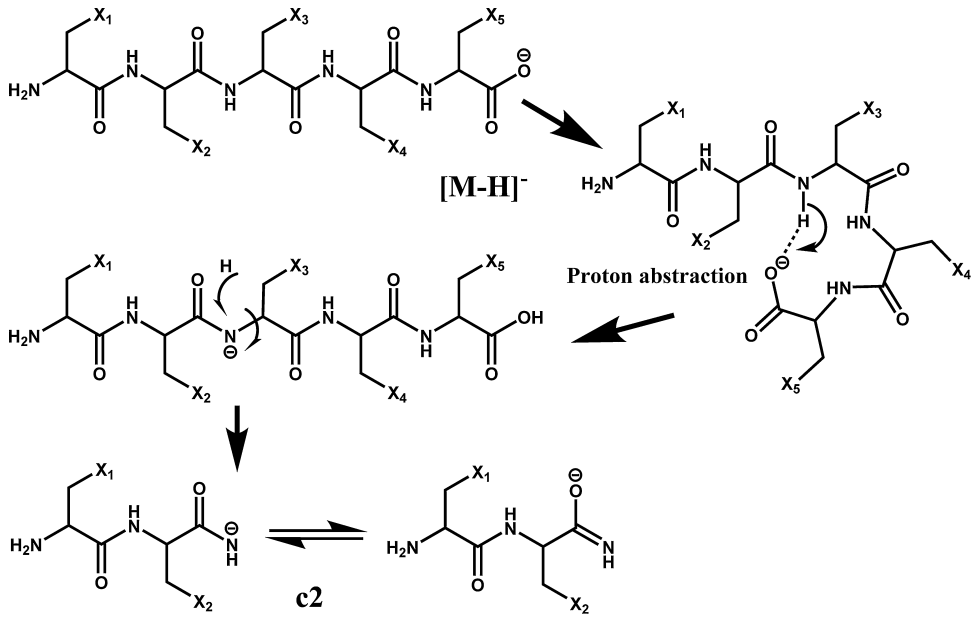
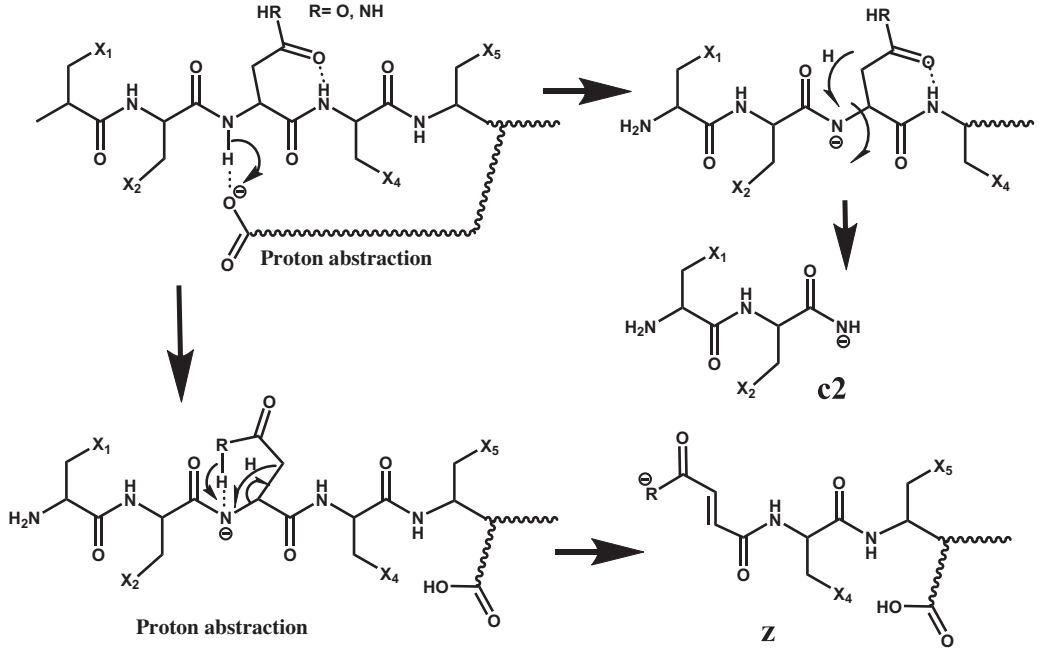


Fig. 3. Negative-ion CID spectrum of singly-deprotonated analyte for lysine pentamer K5 (M_m 658.48).



Scheme 4. The mechanism for the formation of negative c-ions due to intra-molecular proton abstraction via 9-membered ring state proposed by Bokatzian-Johnson et al. [13], in the case of peptides without any acidic or Asn residues.



Scheme 5. Proposed mechanism for the formation of c- and z-ions due to intra-molecular proton abstractions and charge-initiated N—Cα bond cleavage of Xxx-Asp/Asp residues.

because the side chain in Asp/Asn residues can interact with the backbone amide hydrogen and the negatively charged amide nitrogen (Scheme 5). According to the reports of Harrison and Young [12] and Bokatzian-Johnson et al. [13] and the CID result of K5 described above (Fig. 3), it is suggested that the N—Cα bond cleavage to form c-ions is caused by negative charge located on the amide nitrogen, generated with an intra-molecular proton abstraction, as shown in Scheme 4. Furthermore, the formation of z-ions may be explained by a proton transfer from active hydrogens (COOH and $CONH_2$) of Asp/Asn residues to negatively charged nitrogen atom (Scheme 5). The mechanism for the c/z-ion pair formation proposed here needs further study including chemical calculations.

4. Conclusion

In the CID experiments of peptides, the formation of c- and z-type product ions is an unusual event, while b- and y-type ions with and without neutral loss are often observed in both positive- and negative-ion CID spectra. When analyte peptides have Asp and Asn residues, however, the negative-ion CID spectra show dominant c- and z-ions originating from cleavage at the N—Cα bond of Xxx-Asp/Asn residues. In the absence of any acidic amino acid residues, negative-ion CID spectra show dominant c-ion peaks originating from cleavage at the N—Cα bond lying in between the 3rd and 4th residues from the C-terminus of analyte peptides, as already reported by Bokatzian-Johnson et al. [13]. It is reasonable to assume that the N—Cα bond cleavage to form c-ions in both absence and presence of acidic residues is caused by the negative charge located on the amide nitrogen. In the presence of Asp/Asn residues, the formation of z-ions may be due to charge-initiated N—Cα bond cleavage and intra-molecular proton abstraction from active hydrogens of Asp/Asn side chains.

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