International Journal of Mass Spectrometry 306 (2011) 182–186

Contents lists available at ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Effect of proline position on symmetric versus asymmetric fragmentation of doubly-protonated tryptic-type peptides

Alex G. Harrison*

Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, ON M5S 3H6, Canada

ARTICLE INFO

Article history:
Received 13 August 2010
Received in revised form 6 October 2010
Accepted 12 October 2010
Available online 20 October 2010

Keywords:
Proline effect
Tryptic-type peptide
Symmetric cleavage
Asymmetric cleavage
Collision-induced dissociation

ABSTRACT

The fragmentation reactions of the doubly-protonated tryptic-type peptides APAAAAR, AAPAAAR, AAAPAAR and AAAAPAR have been studied using an electrospray/quadrupole/time-of-flight (QqTo F) mass spectrometer. The tendency to cleave amide bonds N-terminal to proline (P) is in competition with the tendency to cleave the second amide bond, counting from the N-terminus; the result of such competition depends on the position of the Pro residue. When the Pro residue is remote from the Arg (R) residue a strong proline effect is observed resulting in formation, to a large extent, of a doubly-charged y species and a neutral fragment, so-called asymmetric amide bond cleavage. By contrast, when the Pro residue approaches the C-terminal Arg residue the proline effect is reduced with respect to cleavage of the second amide bond; in both cases formation of singly-charged y and b ions, so-called symmetric bond cleavage, increases significantly in importance. The results are discussed in terms of relative energetics for symmetric and asymmetric bond cleavage as revealed by approximate proton affinities of the b species and the singly-charged y species.

1. Introduction

Collision-induced dissociation (CID) of protonated or multiply-protonated peptides has become a widely used method for deriving sequence information [1–3]. Sequence determination for proteins often involves digestion by trypsin prior to CID of the tryptic peptides. Trypsin usually cleaves proteins C-terminal to arginine or lysine producing peptides with a highly basic residue, arginine or lysine, at the C-terminus of the peptide. Such basic residues will localize a proton on the basic side chain [4] which often leads to fragmentation at the side chain for singly-protonated peptides rather than cleavage along the peptide backbone which is necessary for sequencing. As a result, fragmentation of doubly- or multiply-protonated peptides, in which there is a mobile proton [4], often provides more complete sequence information.

There have been many mechanistic studies of the fragmentation of singly-protonated non-tryptic peptides; these have been summarized in recent reviews [5,6]. On the other hand much less is known in detail concerning the fragmentation of doubly-protonated species, particularly tryptic peptides. Although there have been several limited mechanistic studies [7–13], equally important information has come from statistical analyses of data bases of tandem mass spectra of tryptic peptides [14–16]. Thus, it has been elucidated [9,15] that such doubly-protonated peptide ions tend to cleave N-terminal to proline residues. Recently, Zubarev and co-workers [16] have elucidated a bifurcating behaviour of doubly-protonated tryptic peptides in that there is a class of peptides where cleavage of the second amide bond (counting from the N-terminus) in charge-separation reactions is clearly preferred; this preference is particularly evident for shorter chain length peptides [11,16].

Doubly-protonated peptides may fragment to form a doubly-protonated fragment and a neutral species or by charge separation to form two singly-charged fragments. This is illustrated schematically in Fig. 1 [12] where it is proposed that either asymmetric doubly-protonated dimers (as-DPD) or symmetric doubly-protonated dimers (s-DPD) may be formed. In the as-DPD both charges are located on one portion leading, upon fragmentation, to a doubly-protonated fragment and a neutral species. By contrast, in the s-DPD each species in the complex bears a charge and, upon fragmentation, two singly-charged fragments are formed. The latter process is expected to have a barrier greater than the endothermicity as a result of a barrier for the reverse reaction due to coulombic repulsion. On the other hand asymmetric cleavage is not expected to have a barrier greater than the endothermicity.

The studies to date of doubly-protonated tryptic peptides have not clearly elucidated the effect of position of the Pro residue on cleavage N-terminal to Pro, the competition between symmetric and asymmetric cleavage nor the interplay between cleavage of the second amide bond versus cleavage N-terminal to Pro. To explore

* Corresponding author. Tel.: +1 416 978 3577.
E-mail address: aharriso@chem.utoronto.ca

1387-3806/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2010.10.015

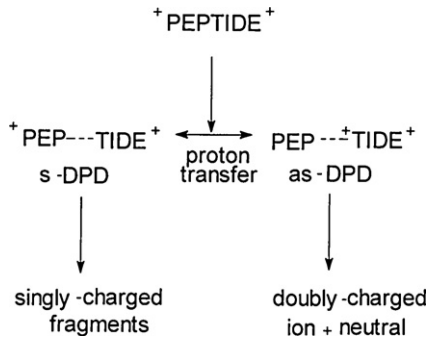

Fig. 1. Schematic of fragmentation routes of doubly-protonated peptides.

these questions in the present work we have studied the fragmentation of doubly-protonated tryptic-like heptapeptides containing Arg at the C-terminus and having five Ala and one Pro in the peptide chain with the position of the Pro residue being varied.

2. Experimental

All experimental work was carried out using an electrospray/quadrupole/time-of-flight (QqTOF) mass spectrometer (QStar XL, MDS Sciex, Concord, Canada). MS/MS experiments were carried out in the usual fashion by mass selecting the ions of interest with the mass analyzer Q followed by CID in the quadrupole collision cell q and mass analysis of the ionic products with the time-of-flight analyzer. In the study of fragment ions (pseudo-MS^3 experiments), CID in the interface region produced fragment ions with those of interest being selected by the quadrupole mass analyzer Q for fragmentation and analysis in the usual manner. By varying the collision energy in the quadrupole cell breakdown graphs expressing, in a qualitative way, the energy dependence of the fragmentation reactions were attained. The cone voltage in the interface region was adjusted to give the best precursor ion signal for the ions of interest; in particular it was found best to use a low cone voltage for transmission of doubly-charged ions.

The peptide samples, at micromolar concentrations, were introduced into the electrospray source in 1:1 CH_3OH:1% aqueous formic acid by a syringe pump at a flow rate of 10 μL/min. Nitrogen was used as nebulizing and drying gas and as collision gas in the quadrupole collision cell. Doubly-charged ion signals were identified by the half-mass separation of the isotopic peaks.

All peptides were obtained from Celtek Peptides (Nashville, TN) and showed no impurities in their mass spectra. Consequently, they were used as received.

3. Results and discussion

Fig. 2 presents the product ion mass spectra for the $[M+2H]^{2+}$ ions of the five heptapeptides APAAAAR, AAPAAAR, AAAPAAR and AAAAPAR recorded at 26–28 eV collision energy (13–14 applied collision voltage). Fragmentation of the $[M+2H]^{2+}$ ion of APAAAAR (Fig. 2a) shows a strong proline effect in that the y_6^{2+} ion is the base peak in the product ion mass spectrum formed by asymmetric bond cleavage at the first amide bond. At the same time there is very significant fragmentation of the second amide bond to produce, by symmetric bond cleavage, the singly-protonated y_5 ion and the complementary products b_2, a_2 and the proline iminium ion Im_P. There is minor formation of the y_4 and y_3 ions along with the b_3 ion counterpart. The y_3 and y_1 ions may be secondary products. The very significant cleavage at the second amide bond is in agreement with the observations of Zubarev et al. [16]. Stein and co-workers [13, Supplementary material] have reported that the $[M+2H]^{2+}$ ion

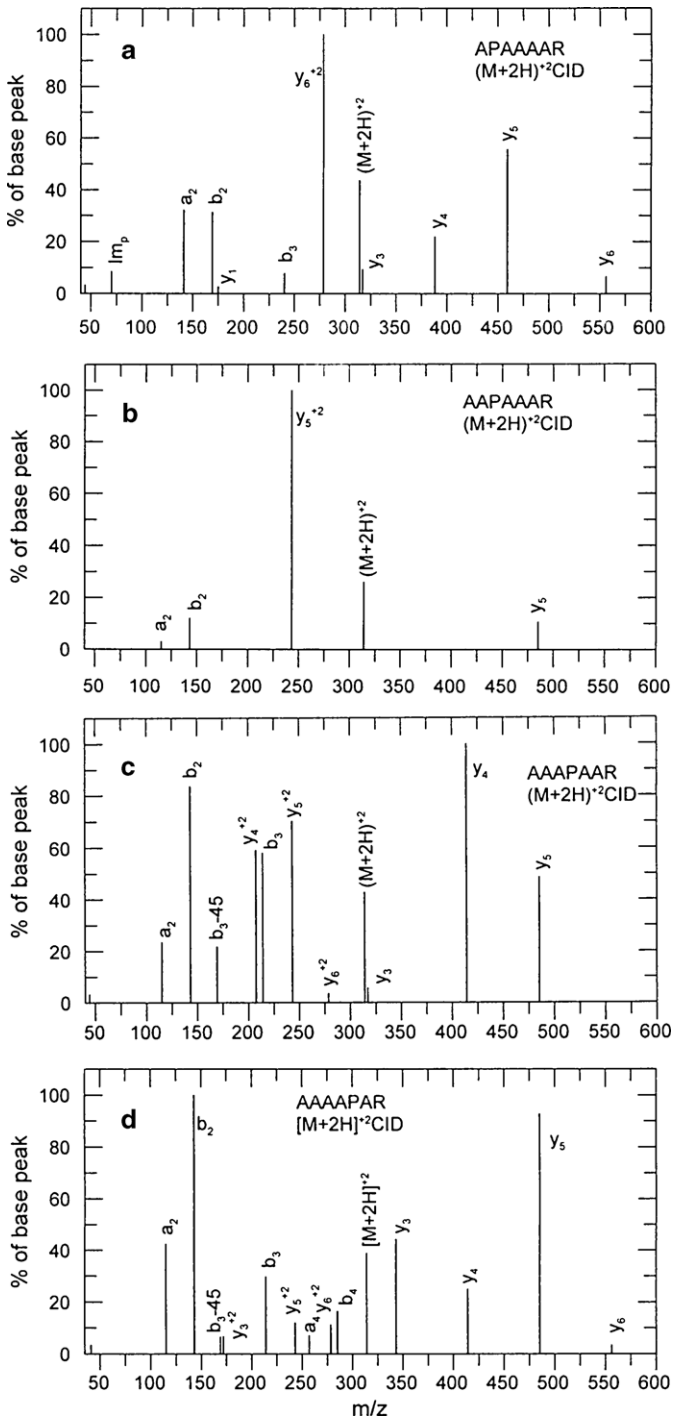

Fig. 2. Product ion mass spectra of $[M+2H]^{2+}$ ions.

of APAAAAK shows y_6^{2+} as the most prominent fragment ion, in agreement with the present work.

Fig. 3 presents the breakdown graph for the y_6^{2+} ion ($[M+2H]^{2+}$ ion of PAAAAR). The dominant fragmentation reactions are symmetric bond cleavage of the first and second amide bonds to form the y_5 and y_4 ions along with the complementary products, the proline iminium ion Im_P and b_2 (and a_2) ions, respectively. The y_1 ion (protonated arginine) is a secondary product, likely arising primarily by further fragmentation of the y_4 ion. In a doubly-protonated $P(A)_xR$ species one proton undoubtedly will be localized on the Arg residue (PA(Arg) = 250.1 kcal mol^{-1} [17]) while the second proton most likely will be associated with the Pro residue since the pro-

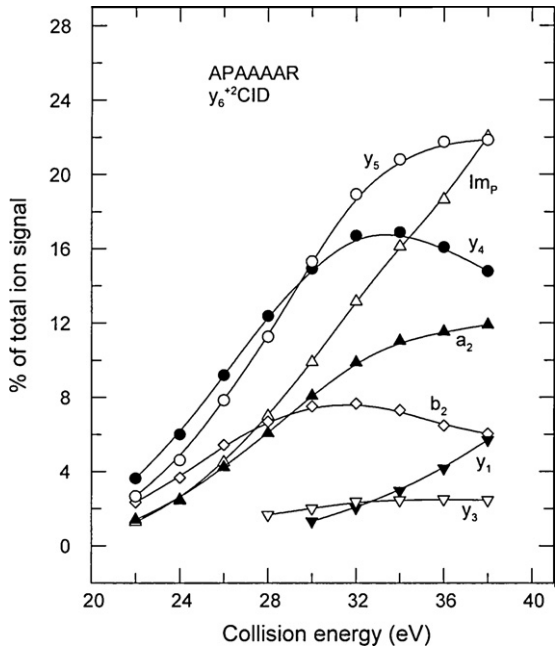

Fig. 3. Breakdown graph for $[M+2H]^{2+}$ ion of PAAAAR.

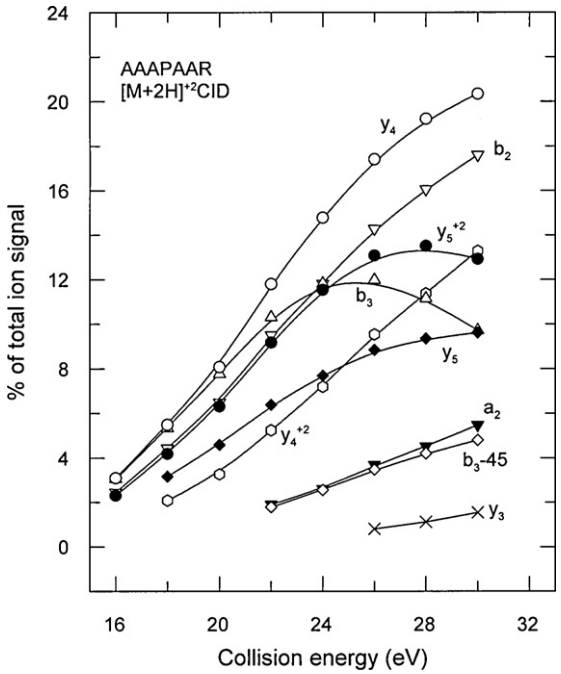

Fig. 4. Breakdown graph for $[M+2H]^{2+}$ ion of AAAPAAR.

ton affinity of proline (225.1 kcal mol^{-1} [17]) is quite high although undoubtedly reduced in the doubly-protonated species by coulombic repulsion.

Since the proton affinity of the Ala imine is only 217.4 kcal mol^{-1} [18] it is not surprising that cleavage of the N-terminal amide bond in doubly-protonated APAAAR occurs almost exclusively by the asymmetric pathway to give y_6^{2+}. On the other hand, cleavage of the second amide bond yields exclusively the AP b_2 ion and the singly-protonated y_5 ion. In this case the second proton affinity of AAAAR is greatly reduced (PA(Ala) = 215.5 kcal mol^{-1} [17] but reduced in singly-protonated AAAAR by coulombic repulsion) while the proton affinity of the b_2 species is enhanced by the Pro residue. The dominant fragmentation reactions of the y_6^{2+} ion involve symmetric bond cleavage to produce singly-charged products. In this case the lower second proton affinities of the Ala-containing y ions makes symmetric bond cleavage least energy demanding. It might be noted that doubly-protonated AAAAAAR fragments almost entirely by symmetric bond cleavage (unpublished results).

The $[M+2H]^{2+}$ ion of the peptide AAPAAAR (Fig. 2b) is the case where the proclivity to fragment N-terminal to Pro and the tendency to cleave the second amide bond coincide. As a result, only cleavage of the second amide bond is observed with the asymmetric cleavage product y_5^{2+} dominating the product ion mass spectrum; there is very minor symmetric bond cleavage to form the y_5/b_2 pair. In this case it appears that the second proton affinity of the PAAAR is greater than that of the AA oxazolone (PA = 222.3 kcal mol^{-1} [19]) or at least very similar given the fact that the barrier for symmetric bond cleavage is greater than the endothermicity because of the coulombic repulsion barrier for the reverse reaction. MS^3 experiments showed that the y_5^{2+} ion ($[M+2H]^{2+}$ ion of PAAAR) fragmented entirely by symmetric amide bond cleavage to give both the y_4 and y_3 products along with the complementary Im_P and b_2 ions. Formation of y_4 (cleavage of the first amide bond) was more prominent than formation of y_3 (cleavage of the second amide bond).

The product ion mass spectrum of the $[M+2H]^{2+}$ ion of AAAPAAR shows (Fig. 2c) that there is substantial cleavage of both the second and third amide bonds, the latter indicative of the propensity for cleavage to occur N-terminal to proline. In both cases significant yields of both singly-protonated and doubly-protonated y products are observed along with the corresponding b ions formed in the symmetric amide bond cleavage. It is clear in this case that symmetric and asymmetric amide bond cleavage are competitive. In cleavage of the second amide bond the significant yield of the y_5^{2+} product would seem to imply that the second proton is associated with the Pro residue rather than the terminal Ala residue. For cleavage of the third amide bond symmetric cleavage becomes competitive both because of the increased proton affinity of the AAA oxazolone (PA = 226.0 kcal mol^{-1} [19]) and the decreased second proton affinity of PAAR due to the proximity of the two charges. The breakdown graph for the $[M+2H]^{2+}$ ion (Fig. 4) shows that both the y_5^{2+} and b_3 ions undergo further fragmentation at higher collision energies. Earlier studies [20,21] have shown that the AAA b_3 ion fragments to form b_3-45 (a_3-NH_3, a_3^*) and the b_2 ion, in agreement with other studies [22] that a_3 ions, in general, are not stable. MS^3 studies showed that the y_5^{2+} ion ($[M+2H]^{2+}$ of APAAR) fragmented to form y_4^{2+} as the major fragment with minor yields of the y_4 and y_3 singly-charged products. Clearly, in this case the second proton affinity of singly-protonated PAAR is sufficient to drive the system to asymmetric amide bond cleavage.

When the Pro residue is close to the basic Arg (R) group, as in AAAAPAR, the proline effect is much less evident. The product ion mass spectrum of the $[M+2H]^{2+}$ ion (Fig. 2d) shows that the major fragmentation mode is symmetric cleavage of the second amide bond to form the y_5/b_2 product ion pair. There is a modest yield of the y_4/b_3 product pair and a slightly enhanced y_3 ion yield, indicating that there still is a minor proline effect. There are only weak signals for doubly-protonated y ions indicating that asymmetric bond cleavage is of only minor importance. The second proton affinity of PAR will be greatly reduced by coulombic repulsion in comparison with species where the Pro residue is further removed from the basic R group.

As discussed above, the product ion mass spectrum of the $[M+2H]^{2+}$ ion of AAPAAAR shows the y_5^{2+} ion as the major product with only minor formation of the y_5/b_2 ion pair resulting from symmetric amide bond cleavage. In this case the relatively low proton affinity of the AA oxazolone makes symmetric amide bond cleavage

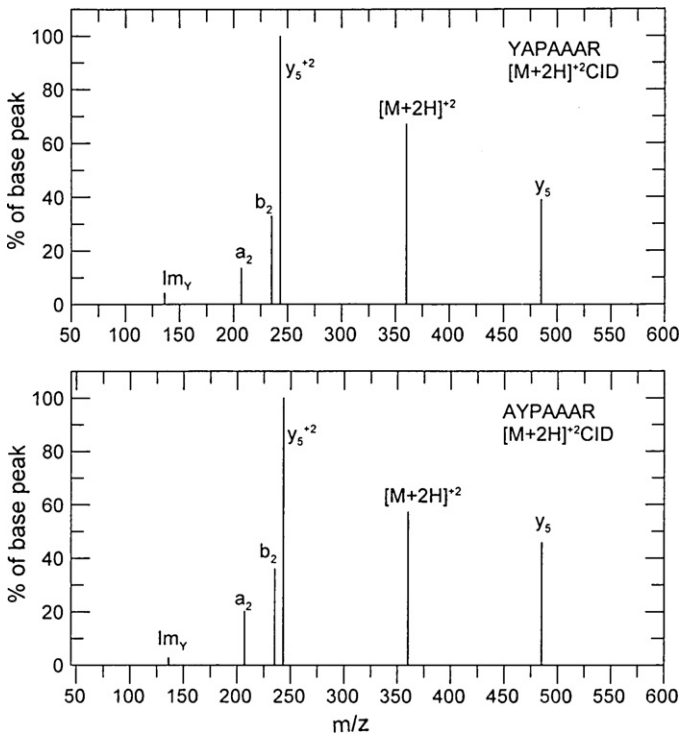

Fig. 5. Product ion mass spectra for $[M+2H]^{2+}$ ions of AYPAAAR and YAPAAAR.

essentially non-competitive with asymmetric amide bond cleavage. Fig. 5 shows the product ion mass spectra for the $[M+2H]^{2+}$ ions of AYPAAAR and YAPAAAR. Although asymmetric bond cleavage to form y_5^{2+} still yields the base peak, the abundance of the y_5/b_2 ion pair formed by symmetric bond cleavage is considerably greater than observed for AAPAAAR (Fig. 2b). Replacing an Ala residue by a Tyr residue has increased the proton affinity of the (presumed) b_2 oxazolone with the result that symmetric bond cleavage increases in importance. It should be noted that Stein and co-workers [13, Supplementary material] have reported that the y_5^{2+} ion is the major product in the fragmentation of the $[M+2H]^{2+}$ ion of AAPAAAK but that the y_5 ion is more abundant than the y_5^{2+} ion in the fragmentation of LLPLLLK and FFPFFFK. The proton affinities of the LL and FF oxazolones would be expected to be considerably greater than that of the AA oxazolone, making symmetric bond cleavage more favourable.

Zubarev and co-workers [16] have proposed that, for those tryptic peptides which show enhanced cleavage of the second amide bond, the b_2 ion formed is a protonated diketopiperazine rather than the more common protonated oxazolone [23,24]. However, more recent experimental results [25,26] concerning the structure of b_2 ions formed from doubly-protonated tryptic peptides have not confirmed this proposal. Table 1 presents the product ion mass spectra for the b_2 ions derived from YAPAAAR and AYPAAAR. (It should be noted that these ions are not produced with any abundance from the singly-protonated peptides so formation from the doubly-protonated species is the most probable origin.) While the spectra are very similar they do not show the products expected for a diketopiperazine structure, such as loss of NH_3, loss of $CO + NH_3$ and loss of $2CO + NH_3$, which have been observed [6,11] in earlier studies of protonated diketopiperazines. The only significant difference is the observation of the m/z 44 ion (Ala iminium ion) for the b_2 ion derived from AYPAAAR. Detailed studies [18,27] of the fragmentation of b_2 and a_2 ions have shown that b_2 ions fragment to a_2 ions and that the latter may fragment to form the iminium ions from both amino acid residues. The results show that the iminium ion of the imine with the greater proton affinity dominates the spectrum and the iminium ion of the imine with lower proton affinity is observed only when it is the a_1 ion. Calculations [18] have derived a proton affinity of 217.4 kcal mol^{-1} for the Ala imine and a proton affinity of 225.1 kcal mol^{-1} for the Tyr imine. In agreement with these calculations the Tyr iminium ion (m/z 136) dominates the product ion mass spectra of Table 1 and the Ala iminium ion (m/z 44) is observed only for the Ala-Tyr b_2 ion. We conclude that most likely the fragmenting b_2 ions have protonated oxazolone structures rather than protonated diketopiperazine structures. However, it remains possible that the stable non-fragmenting structure is a protonated diketopiperazine which rearranges to the oxazolone structure on activation. In this respect infrared multiphoton dissociation (IRMPD) studies represent a preferred approach to elucidating the structures of non-decomposing ions. As noted above, such studies [26] have not confirmed the diketopiperazine structure for b_2 ions derived from doubly-protonated tryptic peptides.

Table 1
CID mass spectra of b_2 ions (14 eV CE).

m/z	Ion	YAPAAAR	AYPAAAR
235	b_2	13.3	4.3
207	a_2	100	100
136	ImY	85.0	82.7
44	ImA		6.1

4. Conclusions

The present study has explored the competition between amide bond cleavage N-terminal to proline [9,15] and cleavage of the second amide bond (counting from the N-terminus) [16] as a function of the position of the Pro residue in doubly-protonated tryptic-type peptides. The results show that there is a strong proline effect, particularly when the Pro residue is remote from the C-terminal Arg residue. Indeed when the Pro residue is in the third position (as in AAPAAAR) the two effects reinforce each other and only cleavage of the second amide bond is observed. In addition there is a competition between asymmetric bond cleavage producing a neutral b fragment and a doubly-protonated y species and symmetric bond cleavage producing a b/y ion pair. The preference for asymmetric cleavage decreases as the Pro residue nears the Arg residue. The results are discussed qualitatively in terms of the relative energetics for asymmetric and symmetric bond cleavage as expressed by approximate proton affinities of the of the relevant species, which also are influenced by the position of the pro residue.

Acknowledgement

I am indebted to the Natural Sciences and Engineering Research Council (Canada) for continuing financial support.

References

[1] M.R. Larsen, P. Roepstorff, Mass spectrometric identification of proteins and characterization of their post-translational modifications in proteome analysis, Fresenius J. Anal. Chem. 366 (2000) 677–690.
[2] R. Aebersold, D.R. Goodlett, Mass spectrometry in proteomics, Chem. Rev. 101 (2001) 269–295.
[3] K.F. Medzihradszky, Peptide sequence analysis, Methods Enzymol. 402 (2005) 209–244.
[4] V.H. Wysocki, G. Tsaprailis, L.L. Smith, L.A. Breci, Mobile and localized protons. A framework for understanding peptide dissociation, J. Mass Spectrom. 35 (2000) 1399–1406.
[5] B. Paizs, S. Suhai, Fragmentation pathways of protonated peptides, Mass Spectrom. Rev. 24 (2005) 508–548.
[6] A.G. Harrison, To b or not to b: the ongoing saga of peptide b ions, Mass Spectrom. Rev. 28 (2009) 640–654.

[7] K. Vékey, M. Candido, P. Traldi, Use of charge separation reactions for sequencing peptides, Rapid Commun. Mass Spectrom. 4 (1990) 74–76.
[8] X. Tang, R.K. Boyd, An investigation of fragmentation of doubly protonated tryptic peptides, Rapid Commun. Mass Spectrom. 6 (1992) 651–657.
[9] J.A. Loo, C.G. Edmonds, R.D. Smith, Tandem mass spectrometry of very large molecules. 2. Dissociation of multiply charged proline-containing proteins from electrospray ionization, Anal. Chem. 65 (1993) 425–438.
[10] X. Tang, P. Thibault, R.K. Boyd, Fragmentation reactions of multiply-protonated peptides and implications for sequencing by tandem mass spectrometry with low-energy collision-induced dissociation, Anal. Chem. 65 (1993) 2824–2834.
[11] A.G. Harrison, Charge separation reactions of doubly-protonated peptides: effect of peptide chain length, J. Am. Soc. Mass Spectrom. 20 (2009) 1890–1895.
[12] M. Knapp-Mohammady, A.B. Young, B. Paizs, A.G. Harrison, Fragmentation of doubly-protonated Pro–His–Xaa tripeptides: formation of b_2^{2+} ions, J. Am. Soc Mass Spectrom. 20 (2009) 2135–2143.
[13] B. Goduku, P. Neta, Y. Simón-Manso, S.E. Stein, Effect of N-terminal glutamic acid and glutamine on fragmentation of peptide ions, J. Am. Soc. Mass Spectrom. 21 (2010) 1169–1176.
[14] D.L. Tabb, L.L. Smith, L.A. Breci, V.H. Wysocki, D. Lin, J.R. Yates, III Statistical characterization of ion trap tandem mass spectra from doubly charged tryptic peptides, Anal. Chem. 75 (2003) 155–1163.
[15] L.A. Breci, D.L. Tabb, J.R. Yates III, V.H. Wysocki, Cleavage N-terminal to proline: analysis of a database of peptide tandem mass spectra, Anal. Chem. 75 (2003) 1963–1971.
[16] M.M. Savitski, M. Fälth, Y.M.E. Fung, C.E. Adams, R.A. Zubarev, Bifurcating fragmentation behavior of gas-phase tryptic peptide dications on collisional activation, J. Am. Soc. Mass Spectrom. 19 (2008) 1755–1763.
[17] S. Gronert, D.C. Simpson, K.M. Conner, A reevaluation of computed proton affinities for the common α-amino acids, J. Am. Soc. Mass Spectrom. 20 (2009) 2116–2123.
[18] A.G. Harrison, A.B. Young, M. Schnoelzer, B. Paizs, Formation of iminium ions by fragmentation of a_2 ions, Rapid Commun. Mass Spectrom. 18 (2004) 1635–1640.
[19] B. Paizs, S. Suhai, Towards understanding the tandem mass spectra of protonated oligopeptides: mechanism of amide bond cleavage, J. Am. Soc. Mass Spectrom. 15 (2004) 103–112.
[20] A.G. Harrison, A.B. Young, Fragmentation of protonated oligoalanines: amide bond cleavage and beyond, J. Am. Soc. Mass Spectrom. 15 (2004) 1810–1819.
[21] A.G. Harrison, Fragmentation reactions of some peptide b_3 ions: an energy-resolved study, Rapid Commun. Mass Spectrom. 23 (2009) 1298–1302.
[22] J.M. Allen, A.H. Racine, A.M. Berman, B.J. Bythell, B. Paizs, G.L. Glish, Why are a_3 ions rarely observed, J. Am. Soc. Mass Spectrom. 19 (2008) 1764–1770.
[23] T. Yalcin, C. Khouw, I.G. Csizmadia, M.R. Peterson, A.G. Harrison, Why are b ions stable species in peptide mass spectra, J. Am. Soc. Mass Spectrom. 6 (1995) 1165–1174.
[24] M.J. Nold, C. Wesdemiotis, T. Yalcin, A.G. Harrison, Amide bond dissociation in protonated peptides. Structures of the N-terminal ionic and neutral fragments, Int. J. Mass Spectrom. Ion Process. 164 (1997) 137–153.
[25] B.J. Bythell, Á. Somogyi, B. Paizs, What is the structure of b_2 ions generated from doubly protonated tryptic peptides, J. Am. Soc. Mass Spectrom. 20 (2009) 618–624.
[26] B.J. Bythell, U. Erlekam, B. Paizs, P. Maître, Infrared spectroscopy of fragments from doubly protonated tryptic peptides, Chem. Phys. Chem. 10 (2009) 883–885.
[27] A.G. Harrison, I.G. Csizmadia, T.-H. Tang, Structure and fragmentation of b_2 ions in peptide mass spectra, J. Am. Soc. Mass Spectrom. 11 (2000) 427–436.