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Why b, y's? Sodiation-induced tryptic peptide-like fragmentation of non-tryptic peptides

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Abstract

Tryptic peptides are the analytes of choice for mass spectrometric analysis of protein and peptide as they display a favorable fragmentation pattern due to the presence of a C-terminal basic amino acid residue. Clean y fragment ion series is most commonly observed for these species. In contrast, non-tryptic peptides with undefined locations of basic amino acid residues give rise to a mixture of b and y fragment ions, often preventing unambiguous assignment of fragment ion types, which in turn impedes the interpretation of the product ion spectra. Here we report that the fragmentation pattern of multiply charged non-tryptic peptides can be modulated by fragmenting the monosodiated multiply charged species instead of the multiply protonated species. Even when b fragment ions dominate the product ion spectrum of the protonated species due to the presence of a charge sequestering basic residue at the N-terminus, mainly singly charged sodium cationized y fragment ions $[y_n + Na]^+$ are observed upon fragmentation of the cationized species, i.e., tryptic peptide-like fragmentation of non-tryptic peptides is achieved.

Several examples of this fragmentation pattern are described, thus strongly suggesting that sodium cation may be complexed near or at the C-terminus even in the presence of other acidic residues within the peptide. This effect is especially pronounced in the case of the doubly charged non-tryptic peptides. This controlled modulation of the fragmentation behavior of non-tryptic peptides is shown to be advantageous for the *de novo* sequencing of non-tryptic bioactive peptides as it facilitates the differentiation between b and y ions.

Keywords: Sodiation; CID; Peptide fragmentation; Non-tryptic peptide; Metal cation

1. Introduction

Tandem mass spectrometry analysis of proteins and peptides represents a favorable alternative to the classical Edman degradation for peptide sequencing, especially for N-terminus modified peptides and complex mixtures. In the mass spectrometric approach, the peptide to be sequenced is selected from a mixture in the first stage of mass spectrometry and subjected to vibrational excitation (activation) typically via collision(s) with gas molecule(s) [1,2] or with surfaces [3] or by absorption of a photon [4,5] with the former being the most commonly applied fragmentation method, also called CID (collision-induced dissociation) or CAD (collisionally activated dissociation). In addition to these slow-heating methods, the fragmentation of peptides could be brought about through non-ergodic processes namely electron capture dissociation (ECD) [6] and electron transfer dissociation (ETD) [7]. All the methods listed above result in peptide activation which subsequently leads to their dissociation. The fragment ions of the dissociation are analyzed in the second stage of mass spectrometry. The resulting MS/MS spectrum is finally interpreted to yield, ideally, the complete amino acid sequence of the peptide.

The most informative dissociations are those that occur along the peptide backbone, i.e., fission of the peptide bond or the C–C bond. Under the conditions of low energy CID (tens of eV), peptides predominantly undergo fragmentation of the peptide bond, i.e., along the peptide backbone. The resulting fragments are denoted using a system of nomenclature that was developed by Roepstorff and Fohlman [8,9] and subsequently modified by Biemann [10] as follows: When a singly charged peptide fragments along the backbone, there are two possible outcomes:

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the charge can be retained either on the N-terminal part, in which case the fragment is designated as a_n, b_n, c_n, or on the C-terminal part to give x_m, y_m, or z_m fragments. The subscripts n and m are used to identify the position of the bond which is broken, counting the number of amino acid residues from either the N- and C-terminus, respectively.

When using tandem mass spectrometry for peptide sequencing, normally it is the protonated peptide species that is selected and dissociated to obtain the sequence information. This is partly due to the long history in mass spectrometry of using protonated species and the ease of protonating peptides. Moreover, the use of trypsin for protein analysis generates peptides with a basic C-terminal amino acid which upon protonation and mass spectrometric fragmentation ensures the formation of a predominant y fragment ion series, thus greatly simplifying the interpretation of the product ion spectra.

Metal cationized peptides are often considered as a nuisance as they (a) reduce sensitivity by distributing the total ion signal over several species, (b) require higher collision energies for peptide fragmentation, and (c) give often suboptimal fragmentation patterns. Samples are, therefore, normally desalted to minimize any salt adduct formation. Nevertheless, salt adducts are commonly observed and hence it would be quite useful if the fragmentation of these, otherwise undesirable, side products could be used to complement the information obtained from the fragmentation of the protonated peptide ions.

A number of research groups have studied the fragmentation of alkali-cationized peptides, $[M + \mathrm{Cat}]^+$ (Cat = alkali metal ions) [11–13] and have shown significant differences in the fragmentation behavior of protonated peptides and their alkali-cationized analogues [14].

While the fragmentation of singly protonated peptides results in the formation of both b and y ions with differing intensities depending on the chemical nature of the peptide, fragmentation of singly charged metal cationized peptides leads predominantly to the formation of a metal cationized b ion $[b_{n-1} + \mathrm{Cat} + \mathrm{OH}]^+$ (where n is the number of amino acid residues in the peptide) as a result of a neutral loss of the C-terminal amino acid residue [11,12]. This particular feature has therefore suggested that metal cationized peptides may be better candidates for stepwise C-terminal sequencing using multiple stages of MS analysis in trapping instruments [15,16].

Several mechanisms were proposed to account for these observations, each results in an OH or O-Cat transfer from the C-terminus (where the metal cation is thought to be bound) [17] to the adjacent amino acid along with the loss of the C-terminal residue from the precursor ion as a CO and an imine. This difference in fragmentation between the protonated and sodiated species could be explained by the structure of the metal adducted peptide ions. Alkali metal ions are thought to interact selectively with polar functional groups to form a chelate coordination structure of the peptide/metal ion complex and can be viewed as fixed (non-mobile) charges of the peptide backbone. Such charge localization is known to lead to charge-remote fragmentation reactions, explaining the observation of fragment ions that still contain the metal adduct. This is in stark contrast to the charge-directed fragmentation typically observed in low energy CID of protonated peptides, where the original fragmentation-inducing charge carrier (the mobile proton) remains with the neutral part of the fragmentation [17,18].

Moreover, a more recent study by Sabareesh and Balaram [19] of the fragmentation behavior of a series of neutral peptides with blocked termini demonstrated that when the metal chelation is restricted to the backbone, the fragmentation of singly charged monosodiated peptides gives rise predominantly to sodium cationized y ions while the fragmentation of their protonated counterparts gives rise to b ions. These findings suggest that the fragmentation of sodium cationized species may indeed yield complementary information to that obtained from the fragmentation of their protonated analogues.

It should be noted, however, that all the studies mentioned above describe the fragmentation behavior of singly charged mono-sodiated non-tryptic peptides. We are not aware of any such a study on multiply charged monosodiated species. In this paper we describe the fragmentation behavior of monosodiated, doubly charged non-tryptic peptides, which is significantly different from their protonated counterparts. Instead of displaying random charge localization upon fragmentation (obvious by mixed b and y fragment ions formation), these monosodiated, multiply charged peptides show distinct tryptic-peptide like fragmentation characterized by dominant $[y_n + \mathrm{Na}]$ fragment ion series.

2. Materials and methods

All chemicals were purchased from Sigma (St. Louis, MO), unless indicated otherwise. High purity, HPLC-grade solvents were from Labscan (Dublin, Ireland) or Burdick and Jackson (Meriden, CT). Synthetic polyalanine peptides were a kind gift from Dr. John Rush, Cell Signaling Technology (Danvers, MA). The peptide ALILTLVS was purchased from Bachem (King of Prussia, PA).

Data were acquired on various generations of the QSTAR quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) including Centaur prototype, QSTAR, QSTAR pulsar, and QSTAR pulsar I with a nanoelectrospray ionization interface (Proxeon, Odense, Denmark). For data interpretation, the (Bio)-Analyst software suite from Applied Biosystems/MDS Sciex (Concord, Canada) was used. Borosilicate capillaries used for nanoelectrospray ionization experiments were purchased from Proxeon.

The crude conotoxin mixture, isolated from *Conus textile* [20], was dissolved in 100 μL of tris-buffered saline (TBS) at a concentration of 10 mg/mL. For the reduction of the cysteine residues, 1 μL of 0.5 M tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was added to 10 μL of the conotoxin solution prior to incubation for 2 h at room temperature. Subsequently, half of the reduced conotoxin mixture was desalted and concentrated on a Poros R2 (Perseptive, Framingham, MA) microcolumn prepared in GELoader tips (Eppendorf, Hamburg, Germany) following described procedures [21,22]. Peptide samples were prepared in 50% aqueous methanol with 5% formic acid and were loaded into borosilicate capillaries.

No sodium salt was added to the peptide solution, the sodium cationized species were a side product, resulting probably from previous steps of peptide synthesis and purification. For the analysis of some of the synthetic peptides, 2 mM sodium acetate was added to the peptides. This indeed increased the relative abundances of the $[M + nH + Na]^{(n+1)+}$ ions, but could also reduce the efficiency of the ionization. Care should therefore be exercised to strike a balance.

3. Results and discussion

The peptide ALILTLVS is commonly used as a calibrant for QSTAR mass spectrometers. One of the features of this peptide is that it shows a dominant signal for the monosodiated doubly charged form even though the monosodiated singly charged species is almost absent. While collisionally activating the $[M + 2H]^{2+}$ and its monosodiated counterpart $[M + H + Na]^{2+}$ peptide for calibration of the product ion mode, we noticed a striking difference in their fragmentation patterns (see Fig. 1A and B) suggesting that the presence of the sodium cation greatly influenced the outcome of the fragmentation. The product ion spectrum of the doubly protonated form showed mostly b and (b-H_2O) fragment ions with minor fragment ions which were assigned as y ions. Such fragmentation behavior is in agreement with the non-tryptic nature of the peptide. The CID of the doubly charged monosodiated species $[M + H + Na]^{2+}$, on the other hand, resulted in the formation of intense sodiated y ions in the *m/z* range above the precursor and a characteristic a_2/b_2 pair in the low *m/z* range. As such this fragmentation behavior seems to mimic the fragmentation of a corresponding tryptic peptide such as ALILTLVR or ALILTLVK.

In order to test whether this observation was specific to this particular peptide or whether it is of a more general nature, we investigated various non-tryptic synthetic peptides as well as non-tryptic protein digest. Our investigations have revealed a significant number of peptides, which exhibit similar patterns of fragmentation upon sodiation, however this is by no means enough to make any generalizations.

Another example is shown in Fig. 2A and B. The product ion spectrum of the triply protonated/charged ACTH peptide

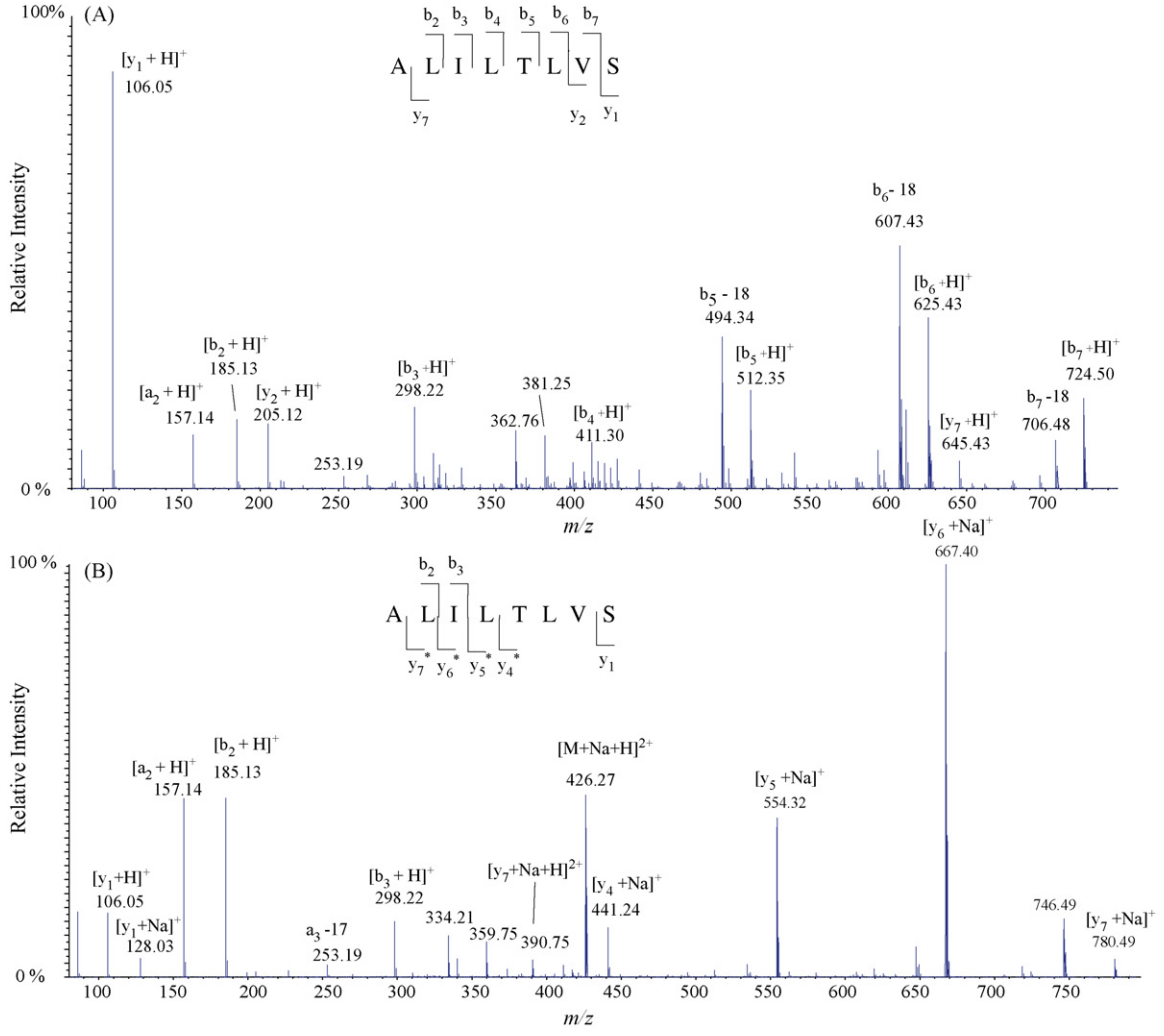

Fig. 1. (A) Low energy CID spectrum of doubly charged peptide ALILTLVS ion at *m/z* 415.27. (B) Low energy CID spectrum of the doubly charged monosodiated peptide ALILTLVS ion at *m/z* 426.27. $[y + Na]^+$ denotes y ions in which a proton has been exchanged for a sodium cation, this nomenclature has been used throughout the paper.

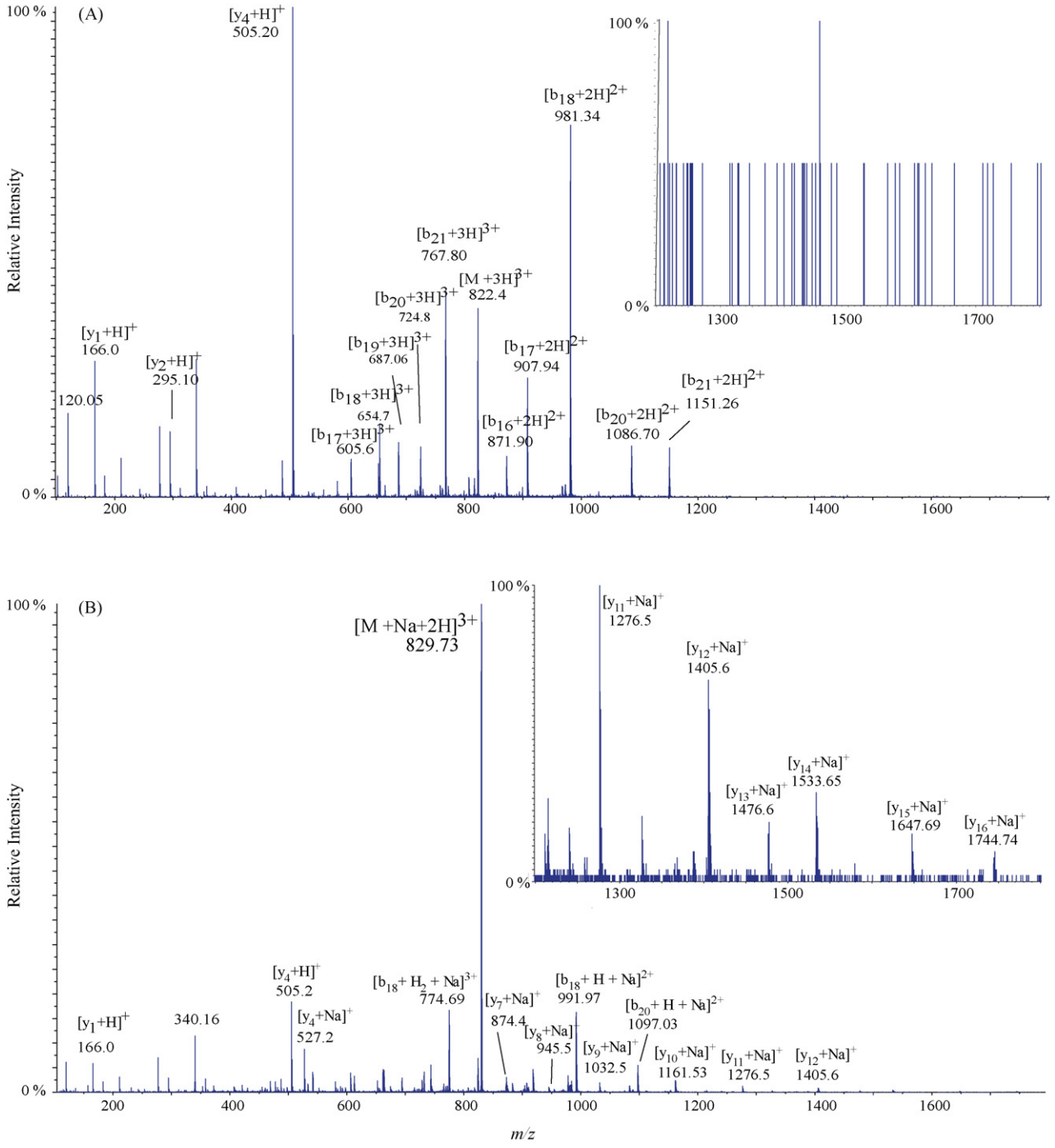

Fig. 2. (A) Low energy CID spectrum of triply charged peptide RPVKVYPNGAEDESAEAFPLEF ion $[M + 3H]^{3+}$ at *m/z* 822.40. (B) Low energy CID spectrum of the corresponding triply charged monosodiated peptide ion $[M + Na + 2H]^{3+}$ at *m/z* 829.73.

(RPVKVYPNGAEDESAEAFPLEF; *m/z* 822.40) is shown in Fig. 2A. As expected for this "inverse" tryptic peptide with two basic residues in positions 1 and 4, we observe a strong $[b_n + 2H]^{2+}$ fragment ion series. In contrast, when fragmenting the monosodiated triply charged species at *m/z* 829.73 we observe several $[y + Na]^+$ fragment ions in the high *m/z*-range between *m/z* 1200 and 1800, corresponding to $[y_{11} + Na]^+$ to $[y_{16} + Na]^+$ where no fragment ions of significant intensity were observed for the non-sodiated species, thereby complementing the sequence information obtained from the protonated species. Not only does this example confirm our previous finding, but it also highlights the fact that the observation might be more generic and not limited to doubly charged species.

This example corroborates the notion that the sodium cation has a tendency to be adducted at the C-terminus of the peptide giving rise to a charge-carrying C-terminus similar to C-terminal arginine or lysine residues in tryptic peptides, thus resulting in the formation of sodiated y ions upon collisional activation. The

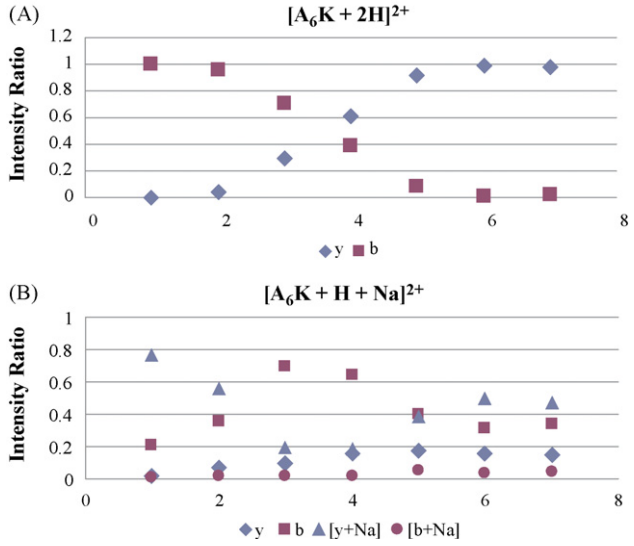

Fig. 3. The distribution of the intensities of the b and y ions generated from the fragmentation of the doubly charged precursor ions as a function of the position and nature of the basic residue. The intensities of the b and y ions were extracted from the product ion spectrum of the doubly charged $[A_6K + 2H]^{2+}$ (A) and $[A_6K + Na + H]^{2+}$ (B).

fact that sodiated b-type fragment ions are also observed could possibly be rationalized by assuming a strong preference of the sodium cation for carboxylic groups in general. As the ACTH peptide contain several glutamic and aspartic acid residues, various subpopulations, which differ in the location of the sodium cation, are conceivable. These different subpopulations could then give rise to sodiated b and y fragment ions. Alternatively, the sodium cation migrates between the different carboxylic groups during collisional activation. More systematic studies on non-tryptic peptides with internal aspartic and glutamic acid residues of charge state three and above are necessary to provide a rationale for this observation.

In order to understand the fragmentation of the sodium cationized species, a more systematic approach was required. A series of polyalanine peptides with the general formula A_6K were synthesized to incorporate K as a basic residue at various positions so as to test the effect that the position of the basic amino acid residue would have on the direction of the fragmentation when comparing fully protonated and singly sodiated species. For each species, the doubly protonated and the monosodiated/doubly charged form were fragmented. Subsequently, we plotted the summed signal intensities of all b and y fragment ions (and their sodium adducts) above the precursor *m/z* value as a function of the position of the lysine residue. The resulting curve for the doubly protonated peptides is shown in Fig. 3A. As can be seen from the figure, when a protonated peptide is fragmented it gives mainly b ions when the basic residue is closer to the N-terminus (K in positions 1–3) and as the position of the basic residue is moved closer to the C-terminus, the peptide fragmentation gives predominantly y ions in agreement with the mobile proton model which suggests that the charge carrier is sequestered by the most basic site (in this case the lysine side chain) [23,24].

In keeping with our previous findings, the fragmentation of the doubly charged monosodiated species was found to be significantly different from the fragmentation of their protonated analogues as shown in Fig. 3B which shows the relative intensity of the b and y ions and their sodiated counterparts. The sodiated y fragment ions $[y + Na]^+$ were the predominant species irrespective of a N-terminal or C-terminal positioning of the lysine residue. This observation corroborates the notion of the sequestration of the sodium cation by the C-terminal carboxylic acid group, thereby giving rise to a preferentially localized charge similar to the basic amino acid residue at the C-terminus of tryptic peptides which promotes the formation of y ions. When the lysine residue was placed in positions 3 and 4 (and 5), higher intensity b ions were observed. This observation is somewhat surprising and its explanation awaits further experiments.

The results shown here suggest that the presence of the sodium cation greatly affects the directionality of the fragmentation, such that the fragmentation of the sodiated peptides gives rise to sodium cationized y ions. This effect may prove useful in inferring peptide sequences especially in the case of non-tryptic, modified peptides, which are otherwise difficult to sequence. The utility of sodium adduction in facilitating peptide sequencing is illustrated for doubly charged monosodiated peptides in Figs. 4 and 5. Both examples were taken from a conotoxin sequencing project, analyzing the crude extract from the venom ducts of *C. textile*. One of the two peptides was chosen for sequencing as it had been identified as tryptophan brominated species based high resolution/high accuracy precursor ion scanning [25].

Fig. 4A shows the product ion spectrum of the doubly protonated peptide at *m/z* 698.7. As expected for non-tryptic peptides, numerous fragment ions were observed; however, the differentiation between b and y ions is not easily achieved. When the monosodiated adduct at *m/z* 709.7 was dissociated a vastly different fragmentation pattern was evident. Since the presence of the sodium adduct promotes the formation of $[y + Na]^+$ ions, we were able to distinguish the y ion series form the b ion series, thus easily deriving the sequence of the precursor ion as XCCYPNVW(Br)CCD (X = I or L), corresponding to the recently reported T-1-conotoxin TeA31 (ICCYPNVWCCD) [26].

Another conotoxin with the molecular weight of 1728.6 Da serves as a second example. When the doubly protonated species at *m/z* 865.3 is subjected to collisional activation, a highly complex fragmentation pattern is observed especially for the *m/z* range above the precursor, thus making unambiguous fragment ion assignments difficult (see Fig. 5A and C). Even the mass accuracy provided by the TOF mass analyzer was not sufficient to avoid ambiguities, such that only partial b and y sequence tags were obtained and ordering them was not straightforward. Sodium cationization of the same peptide almost suppresses the formation of any b ions as can be seen from the product ion spectrum of the monosodiated, doubly charged peptide at *m/z* 876.3 (see Fig. 5B and D). Instead, $[y + Na]^+$ ions become the major type of fragment ions. Moreover, in this particular case an almost complete sodium cationized y ion $[y + Na]^+$ series is observed which allows for the complete *de novo* sequencing of this peptide. Only $[y_1 + Na]^+$ is missing, while the protonated y_1 ion is observed. This observation further corroborates the earlier

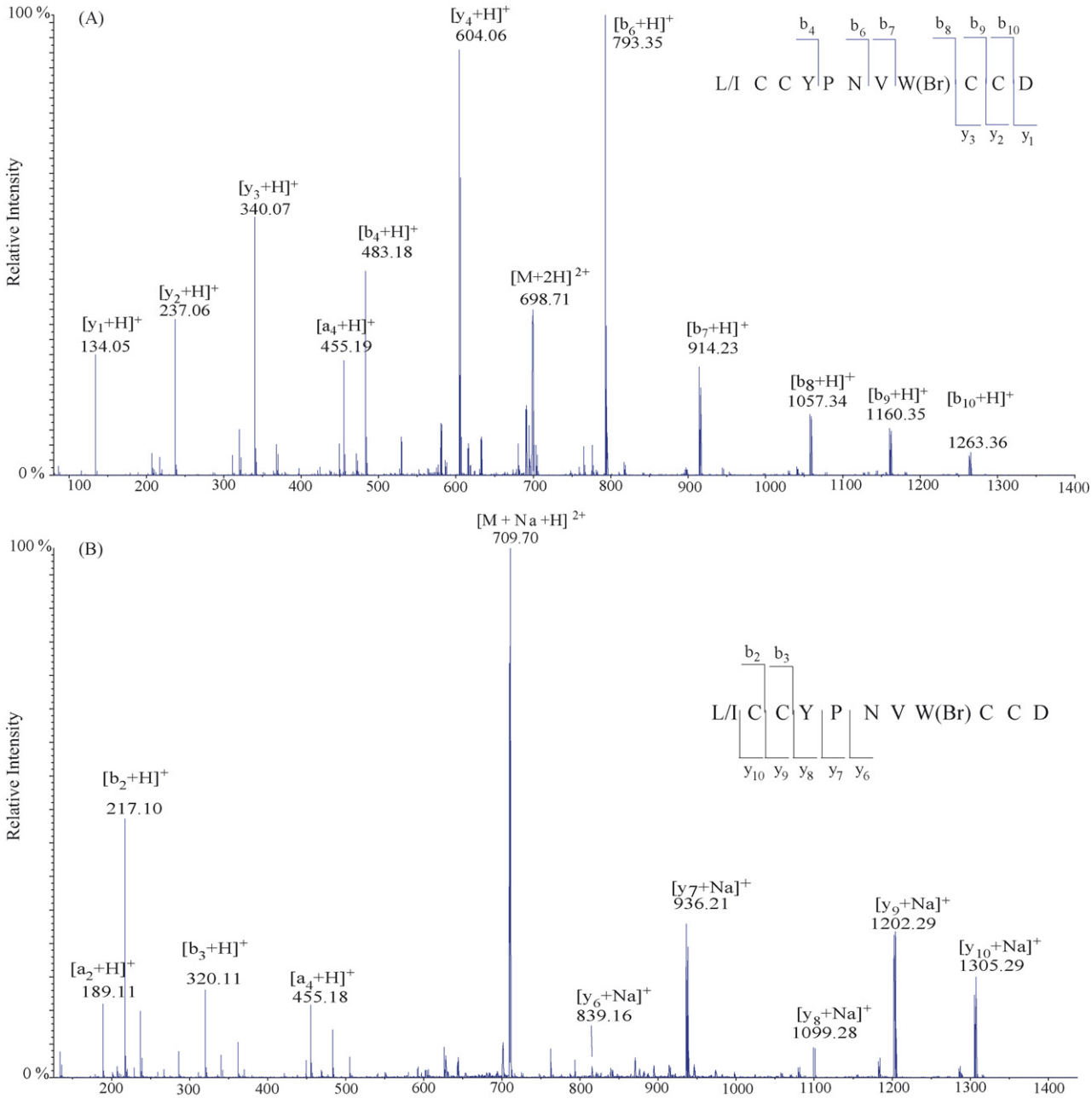

Fig. 4. (A) Low energy CID spectrum of the doubly protonated peptide at *m/z* 698.7. (B) Low energy CID spectrum of the doubly charged monosodiated ion at *m/z* 709.7. The sodium adduction allows the sequence assignment as (L/I)CCYPNVW(Br)CCD.

suggestion that the sodium cation is most likely localized at the very C-terminus.

The sequence VCCPFGGCHELCQCCQ was determined consequently identifying the peptide as conotoxin scaffold III/IV. This sequence could be verified using the fragmentation data from the doubly protonated species. In addition to providing sequence information, our data clearly identifies the C-terminal amino acid as being equivalent to glutamine (*Q*) although a glutamic acid (*E*) residue is expected based on earlier DNA-based sequencing efforts [27]. This indicates that the C-terminus of this particular peptide is amidated (this is a commonly observed post-translational modification in conotoxins) thus identifying the correct sequence as H_2N-VCCPFGGCHELCQCCE-$CONH_2$.

Several aspects of the observed fragmentation behavior of the doubly charged monosodiated species may be explained by the currently used mobile proton mechanism for the induction of peptide fragmentation [23,24]: while the sodium cation is chelated at the C-terminus giving rise to a fairly immobile charge carrier, the second charge carrier, the proton, is sequestered by the most basic site in the peptide sequence. Upon collisional fragmentation, the proton is mobilized, leading to charge-induced fragmentation. This fragmentation is limited to regions farther away from the sodium cation due to Coulomb repulsion, or in other words, peptide backbone cleavage is suppressed close to the location of the sodium cation chela tion.

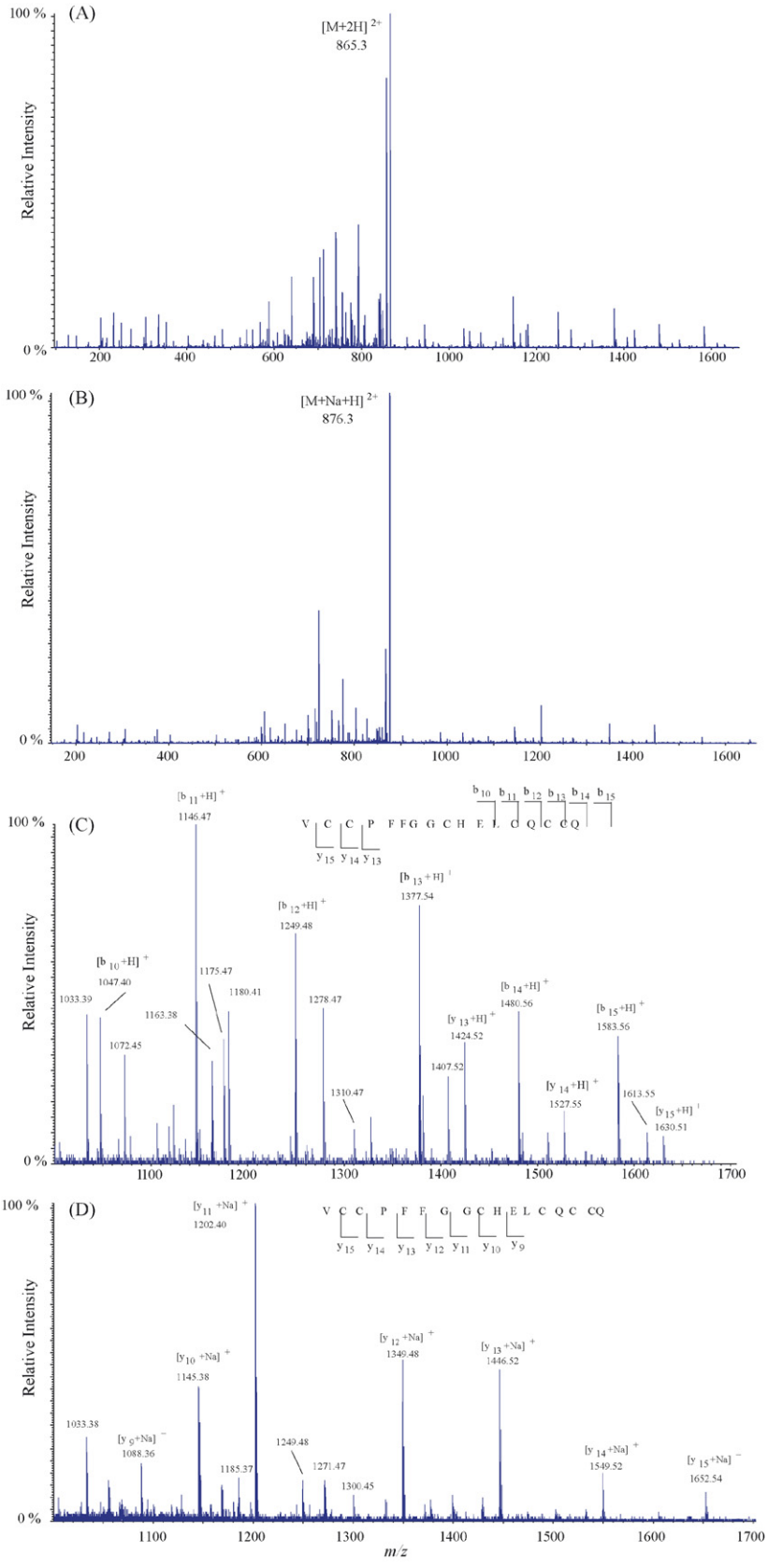

Fig. 5. (A) Low energy CID spectrum of the doubly protonated peptide at *m/z* 865.3. (B) Low energy CID spectrum of the doubly charged monosodiated ion at *m/z* 876.3. The sodium adduction allows promotes the formation of sodium cationized y ions $[y + Na]^+$ and suppresses the formation of high b ions thus allowing the sequence assignment as H_2N-VCCPFGGCHELCQCCE-$CONH_2$.

By assuming that the mobilization of the proton at the ε-amino group of the lysine residue is energetically favored over any mobilization of the sodium cation, numerous described observations can be explained, including the fragmentation $[KAAAAAA + H + Na]^{2+}$ (see Fig. 3). This assumption is, however, only applicable to lysine residue. When we fragmented the monosodiated doubly charged ions of a similar arginine-containing peptide series (RAAAAAA to AAAAAAR), hardly any sequence revealing fragment ions were observed (data not shown). This observation could be rationalized by the higher proton affinity of the guanidine group of the arginine residue that prevents proton mobilization during collisional activation such that no fragmentation-inducing charge carrier/proton is available for arginine containing peptides.

Our observations provide evidence for the collision-induced mobilization of protons from lysine (but not arginine) residues. It is, however, not clear how the location of the lysine residue affects the peptide fragmentation, i.e., the observed distribution of b and [y + Na] fragment ions. While placing a lysine residue at N- or C-terminal positions gives rise to predominant [y + Na] fragments upon fragmentation of the doubly charged monosodiated peptide, the lysine residue in the middle positions can reverse the prevalent fragment ion type (see Fig. 3). Further systematic studies have to be performed for a better understanding of the observation.

4. Conclusions and perspectives

The results presented in this paper suggest that monosodium adduction of multiply charged non-tryptic peptides may direct their fragmentation under low energy CID regime from an undefined distribution of b and y ions to predominantly $[y_n + Na]^+$ ions (and b ions of minor intensity), thereby mimicking tryptic peptides. This modulation of fragmentation can even be observed in the presence of an N-terminal arginine residue that would normally direct the fragmentation towards the predominant formation of b ions. The observed fragmentation behavior strongly suggests that the sodium cation is complexed by the C-terminal or internal carboxylic acid groups leading to the formation of sodiated y ions and protonated b ions. A similar trend, although not as pronounced, was also observed for a triply charged/monosodiated peptide. The data presented in this study also suggest that a lysine-sequestered proton can be fairly easily mobilized during collisional activation which leads to charge-induced fragmentation of doubly charged monosodiated species. In contrast, the higher proton affinity of arginine side chains prevents mobilization of the proton upon collisional activation which, in turn inhibits charge-induced fragmentation.

Although sodiated tryptic peptides are often considered an inconvenience, monosodiation of multiply charged non-tryptic peptides could indeed find some utility in peptide sequencing as it assists in resolving the ambiguity in assigning b and y fragment ions. Once the directionality of the fragment ions is solved, *de novo* sequencing is greatly facilitated as was demonstrated for the two different conotoxins isolated from the crude conotoxin mixture of *C. textile*.

Further systematic studies are, however, necessary to elucidate the mechanism by which sodium cationization affects peptide fragmentation modulation. These studies should include an investigation of different alkali cations, as well as the effect of the presence/absence and position of basic and acidic amino acid residues on the observed fragmentation. Finally, *ab initio* calculations and modeling may also provide some valuable information about the preferential site for metal adduction thus allowing more mechanistic studies to be carried out.

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