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ORIGINAL ARTICLE

Competitive formation of b_2 and c_2-H_2O ions from b_3 ions containing Asp residue during tandem mass spectrometry: the influence of neighboring Arg

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Abstract The fragmentation of b_3 ions derived from protonated Arg-Xxx-Asp-Ala-Ala (Xxx = Ala, Asp, Glu, Cys) and Arg-Xxx-Glu-Ala-Ala was investigated by electrospray ionization tandem mass spectrometry (MS^n) with collision-induced dissociation. A particular ion, which is 1 Da less than b_2 ion, is shown to be the c_2-H_2O ion. The mechanism for its formation involved the aspartic acid in the third position easily losing anhydride to form a c_2 ion, which then lost water to form an eight-membered ring of azacyclooctane derivative under the participation of the guanidine of the N-terminal arginine. However, this phenomenon was not observed when the aspartic acid was replaced by glutamic acid. The Amber program was used to determine the conformation of the original c_2 residue from the dynamic energy perspective, and then density functional theory-based calculations and changing N-terminal amino acid from arginine to phenylalanine supported this mechanism.

Keywords Nontryptic-type peptides · Aspartic acid · Arginine · Theoretical calculations · c_2-H_2O

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Introduction

Mass spectrometry is increasingly important for the analysis and identification of various compounds, particularly proteins and peptides (Larsen and Roepstorff 2000; Aebersold and Goodlett 2001). Peptides are usually ionized by protonation, and they are introduced into a mass spectrometer by electrospray ionization (ESI). Protonated peptides undergo dissociation after collision with an inert gas. The collision-induced dissociation (CID) of peptides is sequence specific, and b and y type ions from the cleavage of the C–N peptide bond (Benjamin et al. 2009). The fragmentation pathways of peptides have been widely studied. Wysocki proposed the mobile proton model of gas phase peptide fragmentation (Donger et al. 1996; Gu et al. 1999; McCormack et al. 1993; Wysocki et al. 2000). This model describes the dissociation in sufficient detail to allow prediction of how likely a given peptide is to fragment in a certain way. Harrison investigated the sequences and possible structures of b ions, and reviewed the structures of b ions with particular emphasis on the way in which structural variation among the fragment ions is influenced by peptide side chains (Somogyi et al. 2012; Harrison 1999; Harrison et al. 2000).

Classical peptide fragmentation pathways are initiated by the addition of proton to the oxygen of carbonyl. Then the proton transfers to the nitrogen of the amide bond, and the protonated amide bond may then be attacked by the oxygen of the N-terminal neighbor amide bond, leading to the formation of a protonated oxazolone derivative (Paizs and Suhai 2005; Yalcin and Khouw 1995; Polce and Ren 2000). Several other types of fragmentation pathway have also been proposed (Balta et al. 2003; Harrison 1999, 2003; Huang et al. 2002; Molesworth and Van Stipdonk 2010; Herrmann et al. 2005). For example, the transfer of the

mobile proton to the nitrogen of the N-terminal amide bond can lead to the loss of the weakly bonded CO via a charge-directed dissociation pathway, which leads in turn to the formation of an a_x ion. Another dissociation pathway involves the side chains of amino acids attacking the C-terminal adjacent to the protonated nitrogen to form cyclic (non-oxazolone derivative) fragments. Their special functions in biochemistry and molecular biology (Jayaraman et al. 2012) cause peptides with acidic (aspartic acid D, glutamic E, and cysteic acid C) or basic (Arg R, Lys K) side chains to be of particular interest (Bythell et al. 2010; Paizs and Suhai 2005; Tsaprailis et al. 1999; Sang et al. 1998; Yu et al. 1993; Gu et al. 2000; Morrison et al. 2012). Especially the peptides which contain aspartic acid have prominent biological function because of its acidic side chain (D'Aneillo and Garcia-Fernandez 2007; Kyathanahalli and Muralidhara 2010). Recently Giuseppina has developed an efficient method for synthesis of long chain diamino acids which can provide the study of biological membranes with the skeleton derived from aspartic acid (Giuseppina et al. 2013). So the research of special fragmentations of peptides contained aspartic acid in gas phase should offer help to the depth study of both biological functions and metabolic products in vivo (Fisher et al. 2007; Zhou et al. 2013; Azevedo 2002). Martin reported that the anhydride is formed upon cleavage of the peptide bond C-terminal to the aspartic acid residue (Yu et al. 1993). Based on that work, Wysocki further characterized the fragmentation of b-type ions formed by aspartic acid in the absence or presence of a mobile proton, and proposed a mechanism in which C-terminal aspartic acid may produce an anhydride structure that can subsequently form d- and c-type ions (Gu et al. 2000; Morrison et al. 2012). Rozman summarized five different mechanisms of selective Asp cleavage, and showed that a side-chain carboxylic group may initiate proton transfer and also bond formation to one of its oxygen atoms (Price et al. 1996; Paizs et al. 2002; Rozman 2007; Wang et al. 2011). Paizs reported the competitive loss of carbon monoxide, water, and ammonia in protonated dipeptides (Pingitor et al. 2004).

This work involved the design of Arg-Ala-Asp-Ala-Ala, Arg-Ala-Glu-Ala-Ala, and other analogous peptides. Tandem mass spectrometry was used to compare the different fragmentations of pentapeptides containing aspartic acid or glutamic acid in the third position. Changes of the second position amino acid were shown to prove the mechanism of the fragmentation; they also allowed assessment of the influence of the acidic side chain (Guo et al. 2010, 2012, 2013; Chai et al. 2012; Zhang and Chai 2012; You et al. 2012). Different N-terminal amino acids were also tested to investigate the influence of arginine. Theoretical calculations (Paizs and Suhai 1998) also supported the mechanism proposed (Harrison 2012).

Experimental

Materials

The peptide targets were bought from ChinaPeptides CO. LTD, and the peptide purity is over 95 % by using RP-HPLC and MS analysis. The sequences of all these eight peptide targets were shown as follow: Arg-Ala-Asp-Ala-Ala, Arg-Ala-Glu-Ala-Ala, Arg-Cys-Asp-Ala-Ala, Arg-Cys-Glu-Ala-Ala, Asp-Glu-Asp-Ala-Ala, Arg-Asp-Asp-Ala-Ala, Arg-Asp-Asp-Asp-Asp-Asp, Phe-Asp-Asp-Asp-Asp-Asp.

Mass spectrometry

Firstly we analyzed these samples in positive ion mode on a Varian 500-MS ion trap mass spectrometer equipped with an ESI source, and used the Varian MS Workstation (Varian, Palo Alto, CA, USA) to achieve the data. The peptides were dissociated in normal CID experiment. Samples for the experiments were prepared by dissolving

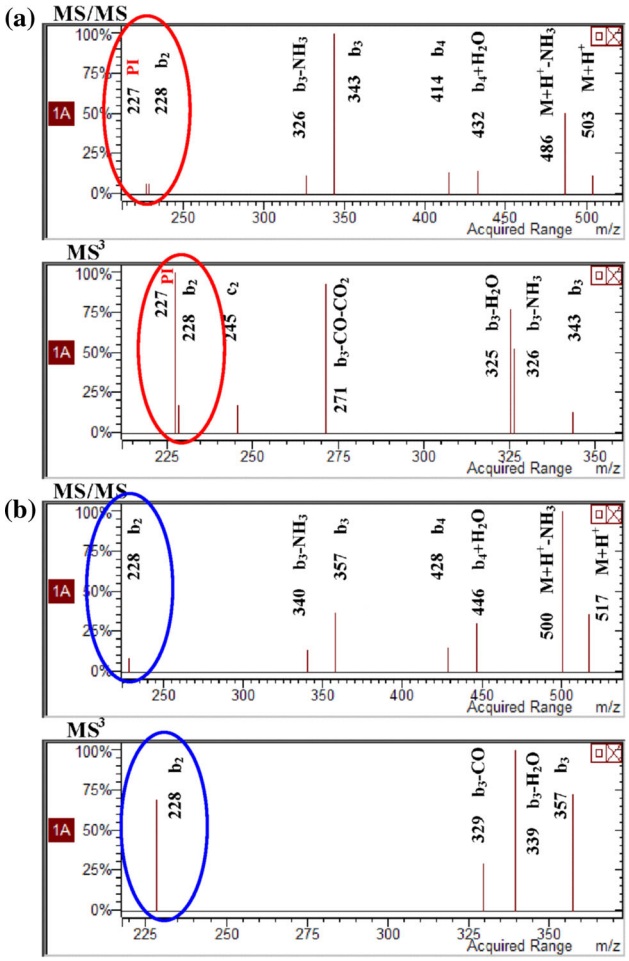


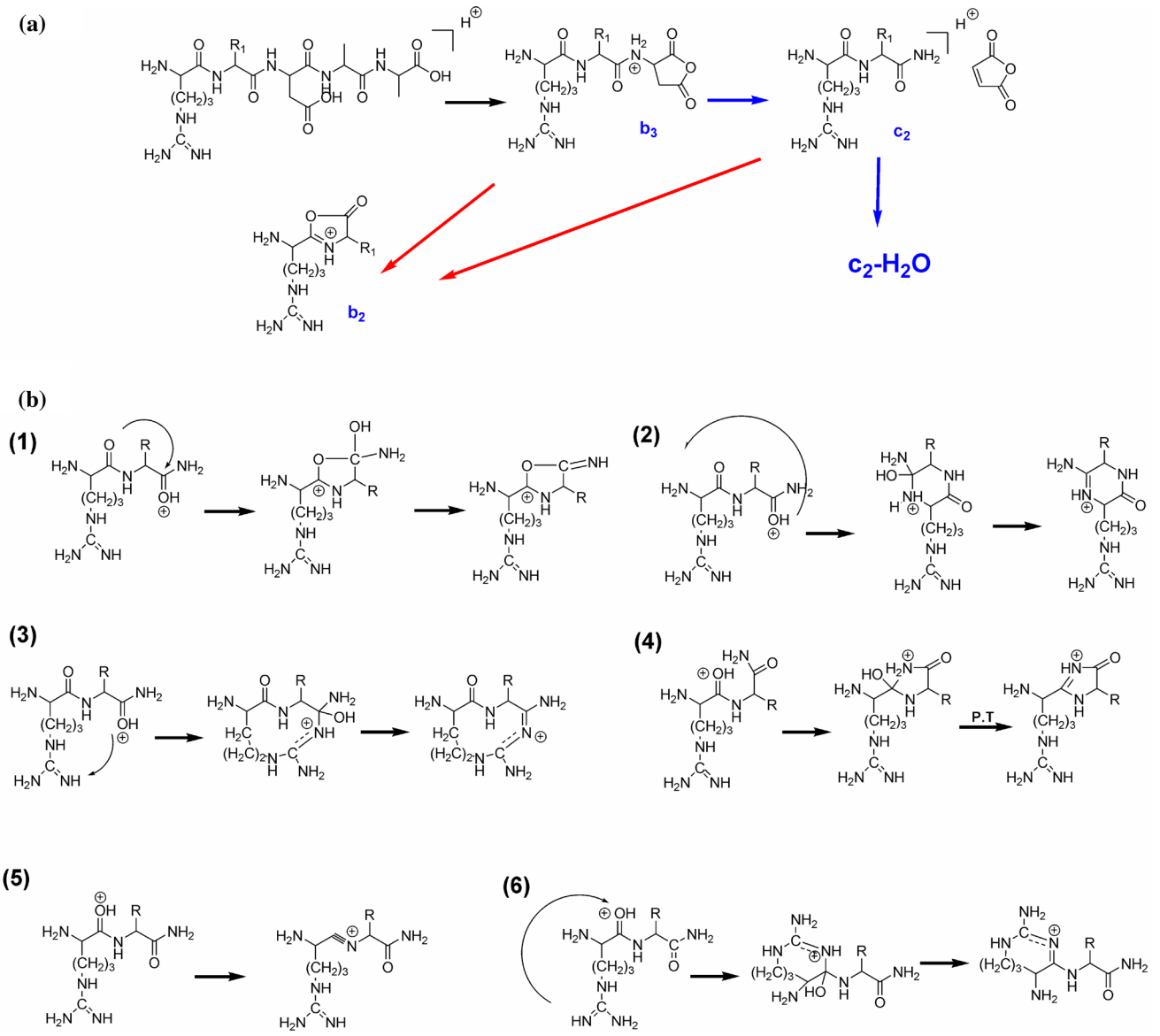
Fig. 1 MS/MS of $[M+H]^+$ ions and MS^3 of corresponding b_3 ions from targets of: **a** Arg-Ala-Asp-Ala-Ala, and **b** Arg-Ala-Glu-Ala-Ala

in methanol/water/acetic acid 50/49.9/0.1 (v:v) to form a 1×10^{-6} mol L^{-1} and infused into the source chamber at a flow rate of 10 μL min^{-1} with the parameters as follow: spray chamber temperature 50 °C, needle voltage 5,000 V, spray shield voltage 600 V, capillary voltage 80 V, RF-loading 80 %, scan mode standard, drying gas temperature 350 °C. Nitrogen was used as the drying gas at a pressure of 15 psi and the nebulizing gas at a pressure of 35 psi. Tandem mass spectra were obtained by CID with helium as the collision gas after isolating the desired parent ion and

Table 1 Relative abundances of product ions in the CID spectra of b_3 ions from six targets (excitation amplitude 0.5 V)

Compound	Sequence	b_3	b_2	b_2-1	b_3-[CO+CO_2]	b_3-Cyclic anhydride
1	Arg-Ala-**Asp**-Ala-Ala	343 (12.8)[a]	228 (97.5)	227 (15.5)	271 (87.5)	245 (17.2)
2	Arg-Ala-Glu-Ala-Ala	357 (72.5)	228 (74.1)	–	–	–
3	Arg-Cys-**Asp**-Ala-Ala	375 (62.4)	260 (9.5)	259 (24.7)	303 (97.5)	277 (9.5)
4	Arg-Cys-Glu-Ala-Ala	389 (11.6)	260 (97.5)	–	–	–
5	Asp-Glu-**Asp**-Ala-Ala	401 (97.5)	286 (22.5)	285 (49.5)	329 (82.5)	303 (11.5)
6	Arg-Asp-**Asp**-Ala-Ala	387 (97.5)	272 (42.3)	271 (44.6)	315 (92.5)	289 (10.7)

[a] *m/z* (relative abundance, %)



Scheme 1 Assumed mechanisms and products from the fragmentation of b_3 residues containing aspartic acid in the third position

the isolation window was set as 1.0 *m/z* unit. The excitation amplitude (resonance mode) was set to give suitable energy for the dissociation of all compounds.

All accurate mass spectrometric experiments were performed on a microTOF-Q II mass spectrometer (BrukerDaltonik, Germany). Bruker Compass Data Analysis software version 4.0 was used for data processing. Sodium formate was used as an external calibration compound. Solutions were infused from the ESI source at 3 μL min^{-1} with parameters: capillary −4,500 V, nebulizing 0.4 Bar, drying gas 2.2 L min^{-1}, drying gas temperature 180 °C. Nitrogen was used as the nebulizing and drying gas, and argon was used as the collision gas. MS/MS analysis was performed through isolation of the desired precursor ion and the collision energy was set at 29.0 eV to give suitable energy for the dissociation of the compounds.

Theoretical calculations

The program of Amber was used to perform the conformational search of the original c_2 ion, which is the parent material of loss of water. By molecular dynamics simulation of 300 ns we divided the series of conformers into ten groups and obtained ten representative conformations. And we also calculated the potential energy by using density functional theory (DFT) method at the B3LYP/6-31+G(d) level of theory in the Gaussian 03 program. Then the global minimum energy of conformation was chosen by comparing both molecular dynamics simulation energy and single point of quantum chemistry energy of ten representative conformations.

The potential energy surfaces were also used to deal with the protonated peptides. The candidate structures of the reactants, products, intermediates and transition states were optimized by calculating the force constants. We started the calculations to modify the initial structures with oxygen- and nitrogen-protonated amide bonds and amide bond-cleavage transition structures. All theoretical calculations were carried out by using the DFT method at the B3LYP/6-31+G(d) level of theory in the Gaussian 03 program. No symmetry constraints were imposed in the optimizations. All optimized structures were subjected to vibrational frequency analysis for zero-point energy correction. The sum of electronic and thermal energies of the optimized structures was discussed (Paizs and Suhai 1998).

Results and discussion

Dissociation of six protonated targets

The tandem mass spectra of the protonated peptides Arg-Ala-Asp-Ala-Ala and Arg-Ala-Glu-Ala-Ala are shown in Fig. 1. The spectra show almost identical fragmentations of these two targets. The N-terminal arginine can easily capture the proton, which favored the formation of b-type ions and the loss of ammonia from the guanidine group. The carboxyl group in the C-terminal led to ions of the types M+H-NH_3 and b_n+H_2O. However, a particular ion of 227 Da resulted from the fragmentation of Arg-Ala-Asp-Ala-Ala. This fragment was identified via the

Table 2 The accurate mass data of b_2 and PI from MS/MS of Arg-Ala-Asp-Ala-Ala on high-resolution mass spectrometry

	Assumed fragment	Exact mass	Actual mass	Error (ppm)
1	b_2	227.1615	227.1623	+3.5
2	c_2-H_2O	228.1455	228.1454	−0.44

Table 3 The comparison of molecular dynamic energy and quantum molecular energy of ten representative conformations from molecular dynamics simulation

File_name	Snapshot (ps)[a]	RMSD (Å)[b]	*E* (MD) (kcal/mol)[c]	*E* (QM) (kcal/mol)[d]
P1	6,466	1.12779	−27.8239	0.00
P2	7,406	1.0418	−30.0962	3.99
P3	9,391	0.92319	−51.9328	−20.85
P4	14,996	0.99239	−37.6665	0.13
P5	30,976	0.93151	−48.2213	−6.10
P6	35,516	1.03706	−53.5949	−1.94
P7	60,026	1.01201	−45.9279	−4.12
P8	60,276	0.96097	−37.6008	2.36
P9	78,931	0.78085	−37.622	−6.39
P10	99,501	1.34668	−48.8649	−8.08

[a] Corresponding to different conformation in 300 ns

[b] Root-mean-square deviation

[c] Energy of molecular dynamic

[d] Energy of quantum molecular

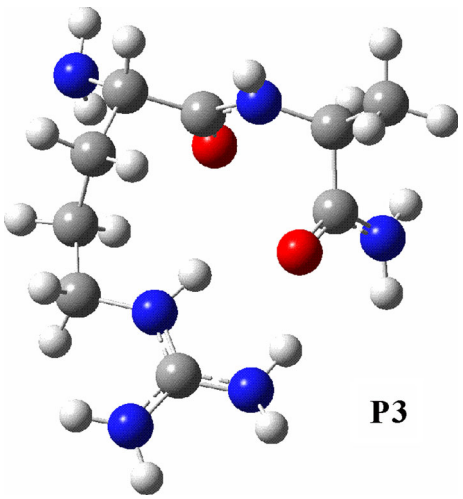


Fig. 2 The conformation structure of original protonated c_2 ion

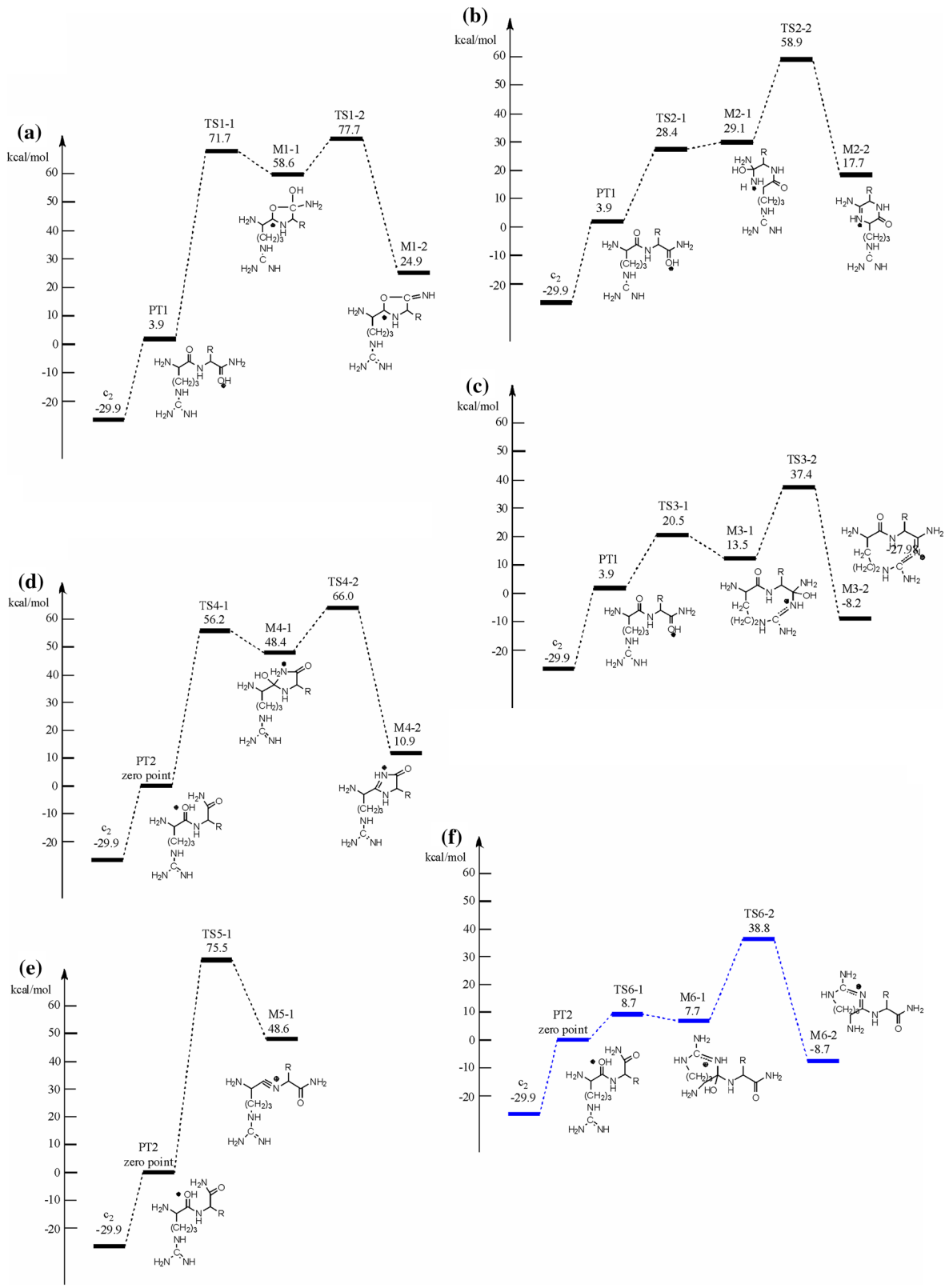


Fig. 3 **a–c** Potential energies of the possible structures of fragments of the c_2-H_2O ion from a **PT1**, calculated by DFT at the RB3LYP/6-31+G(d) level. **d–f** Potential energies of the possible structures of fragments of the c_2-H_2O ion when from **PT2**, calculated by DFT at the RB3LYP/6-31+G(d) level

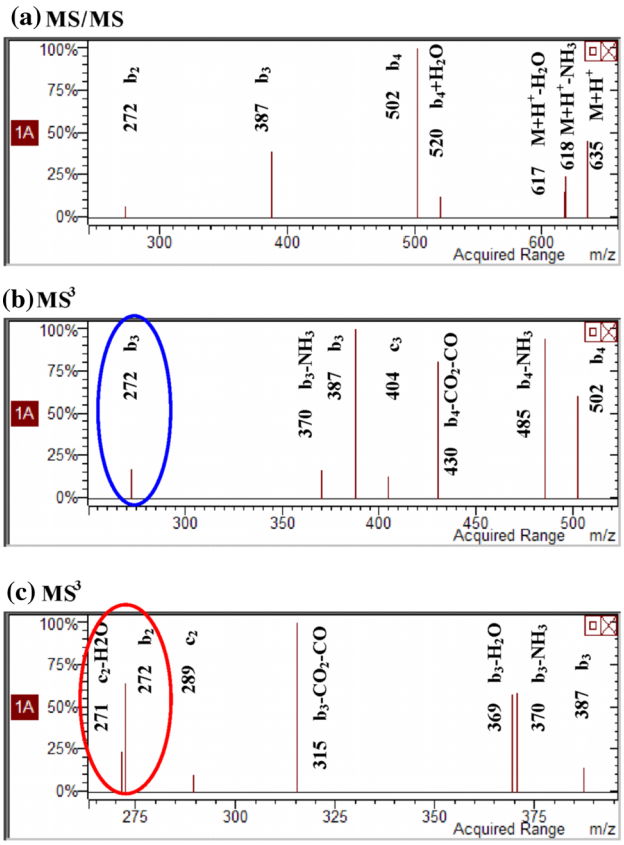


Fig. 4 Dissociation of an Arg-Asp-Asp-Asp target during mass spectrometry: **a** MS/MS of $[M+H]^+$, **b** MS^3 of the b_4 ion, and **c** MS^3 of the b_3 ion

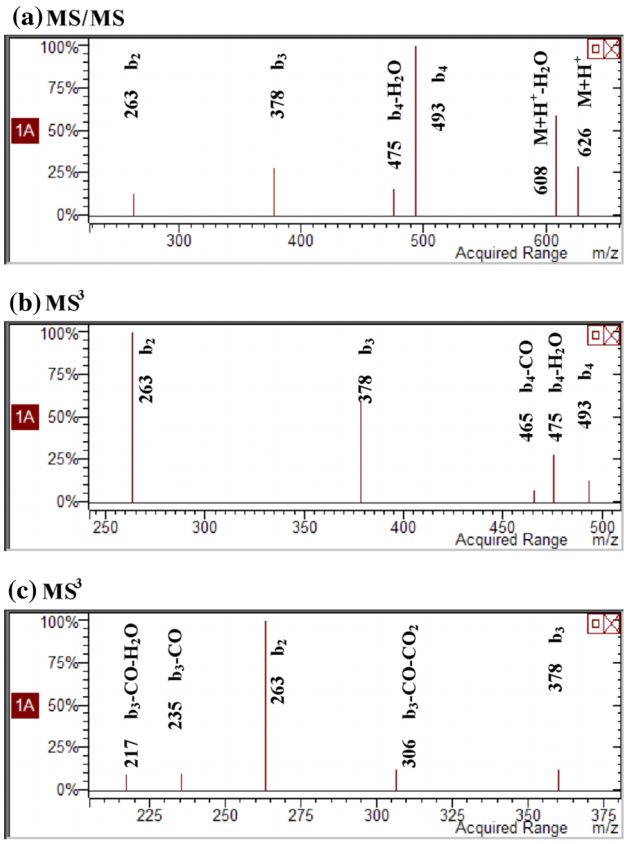


Fig. 5 Dissociation of a Phe-Asp-Asp-Asp target during mass spectrometry: **a** MS/MS of $[M+H]^+$, **b** MS^3 of the b_4 ion, and **c** MS^3 of the b_3 ion

multistage fragmentations of the b_3 ions of the two targets. MS^3 showed very different cleavage patterns for these residues. Aspartic acid in the third position led to losses of 72 Da ($CO+CO_2$) and 98 Da (maleic anhydride) (Fig. 1a), as previously reported (Wysocki et al. 2000). Furthermore, a particular fragment P1 (1 Da less than the b_2 ion) emerged and was much more abundant than the b_2 ion. However, glutamic acid in the third position led to these, the three particular losses not being observed.

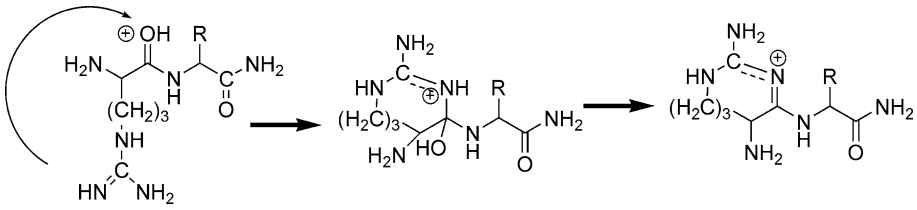
Four correlative targets with different amino acids in the second position were next studied. The fragments observed by MS^3 are summarized in Table 1. The amino acid (Ala, Asp, Cys, or Glu) in the second position did not affect the three particular losses, which all occurred when the third position amino acid was aspartic acid. This phenomenon may allow the differentiation of different peptides containing aspartic acid and glutamic acid.

We next propose a mechanism for the fragmentation of the b_3 residue. When the b_3 ion of aspartic acid residue dissociates, it initially forms a five-membered ring of anhydride. The subsequent loss of $[CO+CO_2]$ or maleic anhydride, which leads to the formation of the c_2 residue, is an easier process than the direct formation of the b_2 ion (Fig. 1; Scheme 1a). This intermediate c_2 can lose water to form c_2-H_2O more easily than it can lose ammonia to form b_2 (Scheme 1a). High-resolution mass spectrometry allowed the identification of the elemental composition of these products and the testing of this assumed mechanism (Fig. S2; Table 2).

Replacement of the aspartic acid with glutamic acid affected the fragmentation: the loss of the whole glutamic acid to form directly a six-membered ring anhydride became easier than the loss of the particular fragments first. Note that water can be lost from the c_2-H_2O residue via several different pathways. The proton first transfers to the oxygen of the carbonyl in the second- or third-position amino acid. The oxygen can then capture the hydrogen from the ammonia to cause the loss of water and the formation of several fragment structures. The carbon of the protonated carbonyl is also attacked by ammonia from the C-terminal or the side chain of arginine to form a C=N bond as the water is lost. Scheme 1b shows the possible mechanisms and the structures of the fragments.

Theoretical calculations

The mechanisms were further explored via theoretical calculations at the RB3LYP/6-31+G(d) level of theory,



Scheme 2 Two possible fragmentation mechanisms of the c_2-H_2O ion. *R* represents the side chain of the second position amino acid

which were used to assess the energy requirements of these reactions. P1–10 is the ten representative conformations we have classified from molecular dynamics simulation of 300 ns and the numerical comparison is shown in Table 3. From these comparisons we have found although the molecular dynamic energy of P3 isn't the lowest (just higher than that of P6). But the quantum molecular energy of P3 is remarkable lowest in ten conformations and by synthesizing these two results we can ensure P3 is the conformation with the minimum potential energy (Fig. 2).

When the proton is transferred to the oxygen of a carbonyl group, it can form either structure **PT1** or **PT2**. The **PT2** was set as the zero point; the energy of **PT1** is 3.89 kcal (Fig. 3). **PT1** can then produce three types of fragmentation (Fig. 3a–c) and **PT2** can also form a further three types (Fig. 3d–f). Among these six types, the third type (Fig. 3f), which loses water via the guanidine side chain of the N-terminal arginine attacking the protonated carbonyl in the second position amino acid, is the most likely fragmentation. This is due to this pathway showing the lowest energy not only of the transition state but also of the middle and final products (Fig. 2).

Further investigations of mechanism

MS^4 analysis of further fragmentations of the c_2-H_2O residue corroborated the theoretical calculations. It was investigated whether the c_n-H_2O ion fragment formed in the case that aspartic acid was also located in other positions (e.g., in the fourth and fifth positions) and in the case that the arginine was exchanged for other amino acids, based on an analysis of targets with the sequences Arg-Asp-Asp-Asp-Asp and Phe-Asp-Asp-Asp-Asp. The former, with addition of aspartic acid in other positions, also showed the particular loss of the $[CO+CO_2]$ ion and the maleic anhydride ion during tandem mass spectrometry. However, aspartic acid must be in the third position for the c_n-H_2O fragment to form. The latter, Phe-Asp-Asp-Asp-Asp, did not fragment into the c_2-H_2O ion, suggesting that the N-terminal amino acid must be arginine for this ion fragment to form (Figs. 4, 5).

A possible mechanism of the fragmentation of the c_2-H_2O ion was identified (Scheme 2). The guanidine of the N-terminal arginine attacks the carbon of the protonated carbonyl to form the c_n ion, and the loss of water then results in an eight-membered ring of an azacyclooctane derivative.

Conclusion

A competitive condensation mechanism was found to govern the fragmentation of the b_3 ions of aspartic acid residues under the influence of N-terminal arginine. The aspartic acid readily forms the five-membered ring of anhydride, which is then lost. The product can subsequently lose water more easily to form the c_2-H_2O ion. This mechanism of water loss also depends on the side chain of the N-terminal arginine. These results suggest the possibility of using tandem mass spectrometry to distinguish aspartic acid from among other acidic amino acids and to identify the position of a small group containing aspartic acid with adjacent N-terminal arginine in long-sequence peptides. This work could form the basis of further study of other distinctive fragmentation patterns that depend on the guanidine side chain of N-terminal arginine.

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Conflict of interest The authors declare that they have no conflict of interest.

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