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Charge-state selective fragmentation analysis for protonated peptides in infrared multiphoton dissociation

Kazuhiko Fukui [a,*], Yasuhide Naito [b,1], Yutaka Akiyama [a], Katsutoshi Takahashi [a]

[a] *Computational Biology Research Center (CBRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-41-6 Aomi, Koto-ku, Tokyo 135-0064, Japan*
[b] *Department of Glycobiology, Tokyo Metropolitan Institute of Gerontology (TMIG), 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan*

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Abstract

We investigate the charge-state selective cleavage in gas-phase protonated peptides by using electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FTICR) mass spectrometer (MS). The singly and multiply protonated peptides (Angiotensin II, Angiotensin I, Urotensin II, Bradykinin, Substance P) at the selected charge state were cleaved with the techniques of infrared multiphoton dissociation (IRMPD) in FTICR MS. Systematically changing IR laser power, the fragment ions were assessed to determine the cleaved amino bonds in the peptides at the selected charge state, then to quantitatively obtain the dissociation efficiency for each fragment. The results show that the fragment ions are observed at the selective cleavage of Asp-Xaa in singly charged Angiotensin I, Angiotensin II and Urotensin II ions that contain an acidic residue (Asp) and a basic residue (Arg or Lys), while the fragment ions arising from cleavage at Xaa-Pro dominate in those doubly charged peptides. It is observed that the dissociation channel of Asp-Xaa requires higher energy than that of Xaa-Pro. The charge selective fragmentation is not seen for Bradykinin and Substance P containing strong basic amino residues without an acidic residue.

Keywords: Peptide; Fragmentation; IRMPD; FTICR MS

1. Introduction

In proteome research, mass spectrometry (MS) has been employed for identification of peptides and proteins in complex mixtures using ion dissociation methods such as collision-induced dissociation (CID) [1–4], infrared multiphoton dissociation (IRMPD) [5–11], surface-induced dissociation (SID) [4,12–14] and electron capture dissociation (ECD) [15,16]. In those methods, CID is the most widely used dissociation method, particularly performed in a low (∼100 eV) collision energy region (low-energy CID). Mass spectrometers with tandem configurations, either tandem-in-space or tandem-in-time, usually incorporate capability of low-energy CID. Using the low-energy dissociation method, protonated peptides are known to yield b- and y-series of fragmentation products [17], and SID is known to be a variant of CID using a collision with a surface instead of neutral gas atoms. In SID, accelerated precursor ions are projected into the solid surface as an incident beam, and fragment ions are collected as the reflected beam. IRMPD employs photons at an infrared wavelength, typically 10.6 μm radiated by CO_2 laser, to activate trapped ions in the gas-phase. Fragmentation properties of IRMPD for protonated peptides are mostly similar to those obtained by low-energy CID. In contrast to the above three methods tending to yield selective cleavages according to the activation regimes, ECD allows protonated peptide precursors to cleave rather randomly in the forms of c- and z-series. The mechanism of ECD is thought to be a non-ergodic process driven by an intra-molecular hydrogen radical, which is generated by capturing a very slow electron. Since a positively charged precursor loses one charge by capturing an electron, this technique is not applicable to singly protonated precursors.

In terms of diversity of cleavages, the fragmentation efficiency of ECD is superior to other activation/dissociation procedures. ECD appears to have the greatest usefulness

* Corresponding author. Tel.: +81-335998060; fax: +81-335998081.
E-mail address: k-fukui@aist.go.jp (K. Fukui).
[1] Present address: Institute of Free Electron Laser, Graduate School of Engineering, Osaka University, Osaka, Japan.

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for de novo sequencing of small proteins [18–20] and for determining post-translational modification sites [21,22]. However, large proteins are still intractable by this method [23,24]. Moreover, precursor ions with a low abundance are not amenable to ECD, because the large fragmentation diversity, i.e., a large number of possible cleavage sites in ECD, may result in a weakness of each fragment peak intensity. On the other hand, low-energy CID and IRMPD have less fragmentation diversities, in turn, the minimum precursor abundance to detect fragments can be much lower than the case of ECD. A highly specific fragmentation obtained by low-energy CID or IRMPD, usually via the lowest activation pathways, may be yet useful to identify small proteins with low quantities, on the ground of an integrated knowledge about the cleaving specificity. The above idea has been emphasized in the splendid demonstration of a doubly protonated N-terminal phenylthiocarbamoyl (PTC) peptide derivative subjected to low-energy CID, which shows exclusive predominance of singly charged fragments associated with cleavage of the N-terminal peptide bond [25]. This procedure can be recognized as an extremely sensitive approach to determine a single N-terminal residue, which is important to protein identification relying on protein databases. Similarly, by defining fragmentation pathways of IRMPD precisely, highly specific cleavages given by IRMPD may bring on a practical merit for database-oriented protein identification. These observations provide quantitative and qualitative clues about the low-energy dissociation channels for the protonated ions.

This study has been motivated by the need to understand sensitivity and selectivity of the fragmentation products obtained from protonated peptides in the gas-phase. Of particular our interest is the combination of electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FTICR) with laser dissociation techniques, such as IRMPD, yielding accurate fragment ion masses with a high resolution far better than that of other mass spectrometers [26]. We investigate the fragmentation properties for standard peptides, Angiotensin II (ANG II), Angiotensin I (ANG I), Urotensin II (URO II), Bradykinin (BRAD), and Substance P (SUB P) at the selected protonation states ($z = 1$ and 2) with ESI FTICR MS.

2. Experiment

2.1. Sample preparation

The Angiotensin II, Angiotensin I, Bradykinin and Substance P were obtained from Sigma (St. Louis, USA), and Urotensin II was supplied by PEPTIDE Institute, Inc. (Osaka, Japan). The entire peptides were used without additional purification. Forty nanomoles of the peptide was dissolved in a 1 ml of HPLC grade 50:50 methanol/water mixture to give a concentration of 4×10^{-5} mol/l. Then, an aliquot was diluted, resulting in a final concentration of 4 pmol/μl. For IRMPD experiments, all solutions were infused into the ESI source using a syringe pump.

2.2. IRMPD experiment with ESI FTICR MS

A 4.7 T ESI FTICR mass spectrometer (BioAPEX II, Bruker Daltonics Inc., Billerica, MA) at Tokyo Metropolitan Institute of Gerontology was used for the IRMPD experiment. The configuration of ESI FTICR MS in detail is described in reference [27]. The accurate mass determination of the peptide fragment ions was carried out without internal calibrants. The mass accuracy achieved by an external calibration using poly-ethylene glycol 1000 was less than 2 and 0.8 ppm in average. The typical mass resolution for IRMPD products was 50,000. The mass range was set to achieve the mass resolution in the broadband detection mode: from *m*/*z* 200 to the mass of singly charged precursor in each spectrum. The original ESI source (original equipment manufacturer: Analytica of Branford Inc., Branford, CT) was modified by an in-house-made microspray apparatus based on a metal-coated fused silica needle (SilicaTips, i.d. = 30 μm, New Objective Inc., Woburn, MA). ESI infusion was performed using a syringe pump at the rate of 0.2 μl/min. Spraying was established by a capillary voltage of 1 kV without an assist gas flow. A TEA-CO_2 laser (Series 48-2, Synrad Inc., Bothell, WA) was placed on the axis of the FTICR ion trap, just outside of the analyzer vacuum chamber. The laser was continuously operated by a pulse width modulation (PWM) waveform at 5 kHz (200 μs period). The laser power level can be controlled by varying the pulse width (PWM duty cycle) at the fixed modulation frequency. The laser was on stand-by while a pulse width of the PWM waveform was 1 μs (tickle pulse). For instance, a 190 μs pulse (95% duty cycle) results in 20 W, which is the maximum power level. The gated operation of the laser is achieved by switching the PWM waveform between the tickle only (gated off) and a larger duty cycle (gated on). Gating and power controlling was performed by using a controller unit (UC-1000, Synrad Inc.), which accepts a user's on/off command pulse and allows IRMPD events to be included in the FTICR pulse sequence generated by Bruker XMASS program. The IR laser beam, whose wavelength was 10.6 μm and a diameter was estimated to be 3.5 mm, was introduced into the vacuum chamber via a ZnSe window without focusing. Since the divergence of the laser beam is 4 mrad, the beam diameter at the FTICR ion trap is estimated to be ca. 5 mm. To investigate the *m*/*z* selective fragmentation, the singly and doubly protonated parent molecules were isolated in the FTICR ion trap using correlated harmonic excitation fields (CHEF) [28]. The fragmentation was investigated at irradiation time from 0 to 200 ms with the laser power in the range from 0 to 20 W, which was determined by measuring the PWM duty cycle with a digital oscilloscope. Taking advantage of the sensitivity and adaptability of FTICR MS, fragment ions of the peptides were screened in the range from 200 to 3000 Da.

3. Results and discussion

In our previous work, we have investigated Angiotensin II in ESI FTICR MS with a CO_2 laser to examine the dependence of fragmentation efficiency on irradiation time from 0 to 1000 ms varying the laser power. We found that abundance of the product ions was virtually unaffected by a function of irradiation time at low laser power, in contrast the fragment ions was significantly increased at high power laser. As a series of our IRMPD study, we here perform the fragmentation analysis of peptides as a function of laser power at the irradiation time (100 ms) using ESI FTICR MS combined with CO_2 laser. Since further absorbing photons cause sequential fragmentation, i.e., fragments originating from internal cleavages, the range of the laser power and the irradiation time are adjusted to avoid sequential fragmentation of primary products of IRMPD. The laser power is the controlling factor for the abundance of IRMPD products at the selected charge state.

3.1. Angiotensin II

Fig. 1 shows the ESI FTICR IRMPD spectrum for Angiotensin II (amino acid sequence: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) with CO_2 laser power of 15.0 W at irradiation time of 100 ms. The mass spectrum of 4 pmol/μl ANG II solution shows the presence of singly, doubly, and triply protonated ions, identified by resolving the isotopic peak spacing. The most abundant ion produced by electrospray was a singly charged peptide ion at $m/z = 1046.562$ Da corresponding to $[\text{ANG II} + \text{H}]^+$. The higher mass peak appearing at $m/z = 1069.552$ Da was the singly charged peaks for the sodium-attached peptide $[\text{ANG II} + \text{Na}]^+$. Dominant fragments were observed from the cleavage in the parent ion at Asp-Arg assigned as y_7 type and at His-Pro as y_2/b_6.

In order to investigate the selectivity of cleavage for producing fragment ions at the charge state ($z = 1$ or 2), we performed CHEF experiments using FTICR MS. Fig. 2(a) and (b) shows the FTICR spectra of IRMPD fragment ions produced from the singly and doubly protonated precursor ions. The figures clearly show the y_7 peak generated from the singly charged parent ion and y_2/b_6 peaks from the doubly charged ion, indicating the selective cleavage at the selected charge state. Controlling CO_2 laser power, IRMPD efficiency curves were quantitatively obtained from the spectra for determining which bond can be more effectively and easily cleaved in the protonated ions. The laser power was determined by measuring the PWM duty cycle with a digital oscilloscope. The dependence of fragmentation on CO_2 laser power for the charge states of ANG II solution are shown in Fig. 3(a) and (b). The relative intensity of y_2/b_6 pair ions cleaved at His-Pro gradually increases when the laser power increases, but the intensity of y_7 type ion is not smoothly increased. The figures show that the required laser power to cleave the peptide bond of His-Pro and Asp-Arg at the irradiation time of 100 ms is 4.1 and 9.2 W, respectively. This result means that the lowest dissociation channel of the protonated Angiotensin II is the peptide bond at His-Pro in $[\text{ANG II} + 2\text{H}]^{2+}$. Comparing the relative abundance of the fragment ions in Fig. 3(a) and (b), the magnitude of the abundance of fragments (y_2) produced from the doubly charged ions are about five times larger than those fragments (y_7) from the singly charged ions. It is worth noting that the peak $y_7{}^{2+}$ found in Fig. 1 is not observed in Fig. 2(b). The dissociation channel of Asp-Arg ($y_7{}^{2+}$) to produce the doubly charged fragment requires much more energy than that of His-Pro(y_2/b_6).

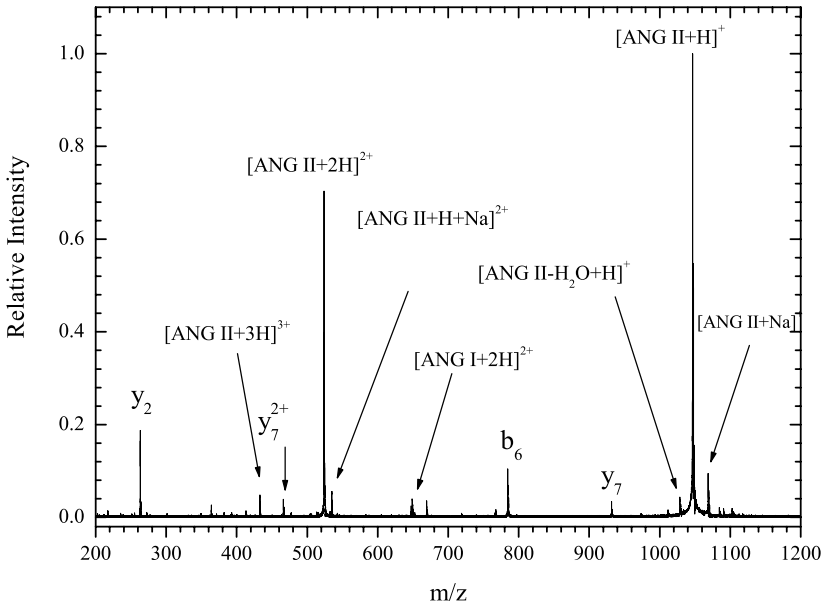

Fig. 1. ESI FTICR mass spectrum of Angiotensin II at the irradiation time of 100 ms with the CO_2 laser power of 15.0 W.

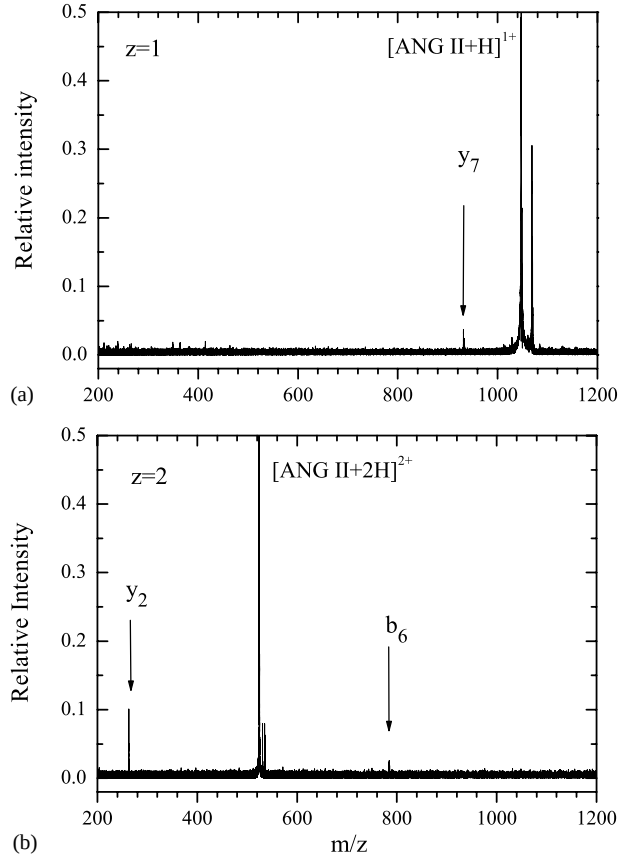

Fig. 2. ESI FTICR mass spectra of (a) singly charged and (b) doubly charged Angiotensin II at the irradiation time of 100 ms with the CO_2 laser power set at 11.4 and 10.0 W, respectively.

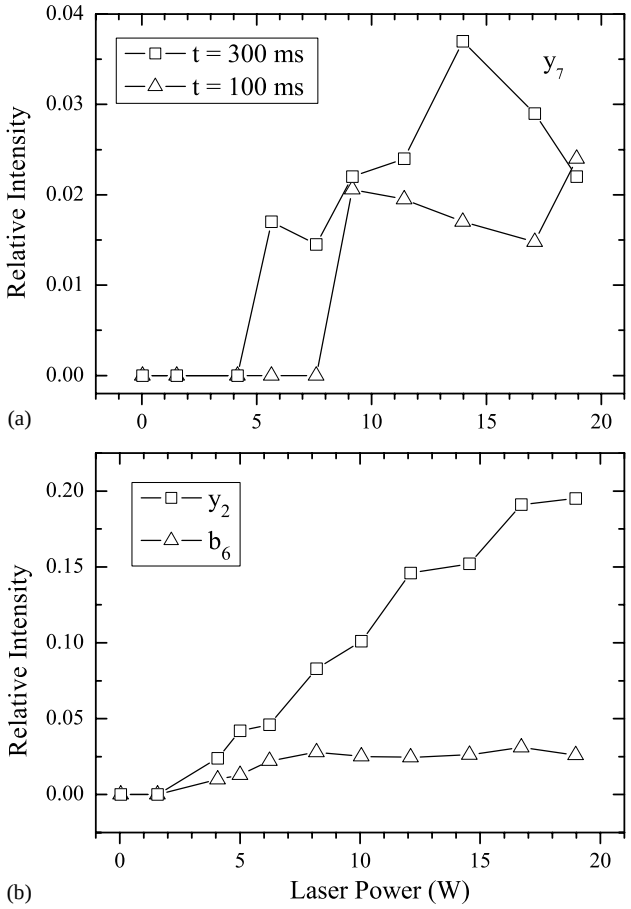

Fig. 3. Dependence of fragment ions on CO_2 laser power in the range from 0 to 20 W obtained from ESI FTICR mass spectra. The panels (a) and (b) show the IRMPD efficiency curves for singly and doubly charged Angiotensin II. The irradiation time is set at 100 ms (triangle)/300 ms (square) for the singly charged ion (a) and 100 ms for the doubly charged ion (b).

Experimental observations of the selective cleavages have been reported for the amide linkage at the peptide bond C-terminal to an acidic residue (Asp-Xaa, Glu-Xaa) [4,13,29–33] or N-terminal to a proline residue (Xaa-Pro) [2,3,34]. It is well known that proline residues have a pronounced effect on chain conformation and the process of protein folding. The rich abundance of y_2/b_6 product ions formed by amide bond cleavage between His and Pro residues is due to proline structural features which are absence of amide protons participating in hydrogen bond and presence of rigid cyclic structure establishing distinct conformational restrictions. Proline-directed fragmentation process in ESI CID experiments, known as proline effect resulting in an abundant y type ion, have been observed by Loo [2] and Vaisar et al. [3].

3.2. Angiotensin I

Fig. 4(a) shows the product ion spectrum of the doubly charged $[ANG\ I+2H]^{2+}$ (amino acid sequence: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) ion with CO_2 laser power of 11.5 W at irradiation time of 100 ms. The dominant observed peaks for the doubly charged solution are b_6/y_4 and b_8/y_2 pair ions corresponding to the cleavage at His-Pro and Phe-His, respectively. It is of interest that the doubly charged fragment b_9^{2+} and y_9^{2+} ions caused by cleavage of His-Leu and Asp-Arg are clearly seen in the IRMPD spectrum (see Fig. 4(a)), while the singly charged fragment ions (b_9^+ and y_9^+) arising from those cleavages were not observed. In the case of fragmentation of the singly charged $[ANG\ I+H]^+$, the peak of y_9 ion is only observed (selective cleavage at Asp-Arg). The dependence of fragmentation on CO_2 laser power in $[ANG\ I+2H]^{2+}$ is presented in Fig. 4(b). The laser power to observe the fragment peaks cleaved at His-Pro(b_6/y_4)/Phe-His(b_8/y_2) in the doubly charged ion and at Asp-Arg(y_9) in the single charged ion is 3.9 and 5.2 W, respectively. This is attributed to the lowest energy dissociation channels at His-Pro and Phe-His in the doubly charged precursor ion.

Tsapralis et al. have investigated the preferential fragmentation pathways for peptides containing both acidic (aspartic, glutamic, or cysteic acid) and basic (arginine) residues with ESI SID experiments [13]. Arginine and lysine residues are know as the strong basic amino acid which side chain

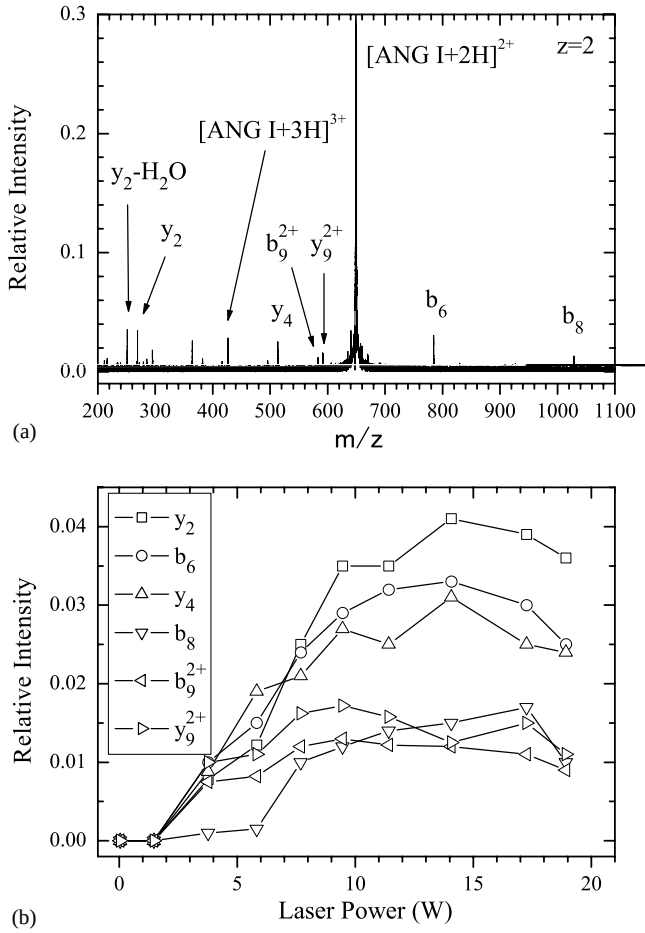

Fig. 4. (a) ESI FTICR mass spectrum of doubly charged Angiotensin I at the irradiation time of 100 ms with the CO_2 laser power of 11.5 W. (b) Dependence of fragment ions for doubly charged Angiotensin I on CO_2 laser power in the range from 0 to 20 W obtained from ESI FTICR mass spectra of Angiotensin I at the irradiation time of 100 ms.

can be the probable protonation sites for the charged parent ion. They proposed a mechanism for the formation of y-type ions at Asp-Xaa linkages with arginine located on the C-terminal side of Asp, considering the five-membered cyclic intermediate in the presence of a fixed charge [35]. In our IRMPD experiments, the fragment ions in singly charged $[ANG\ I + H]^+$ and $[ANG\ II + H]^+$ containing one strong base (Arg) and one acid (Asp) are caused by cleavage of the peptide bond of Asp-Arg, while the fragment ions in $[ANG\ I + 2H]^{2+}$ and $[ANG\ II + 2H]^{2+}$ are dominant by proline-directed fragmentation process. The results are consistent with their ESI SID experiment: selective cleavage at Asp-Xaa with one strong base in a singly charged peptide.

3.3. Urotensin II

The ESI FTICR IRMPD spectrum and efficiency curves for the doubly charged Urotensin II (amino acid sequence: Glu-Thr-Pro-Asp-Cys-Pro-Trp-Lys-Tyr-Cys-Val) are shown in Fig. 5(a) and (b). The spectrum shows that the dominant

ours. Although there is no sign of cleaving the bridge in our result, several groups have established the use of IRMPD for dissociation of protein ions containing Cys-Cys bridges. In particular, IRMPD has been combined with ECD, which alone does not work for the proteins [24,37,38]. To cleave the bridges, higher laser power may be needed rather than that to cleave peptide bond of the lowest dissociation energy. In case of the peptides that contain S–S bridges, high energy CID is more effective than IRMPD to determine a variety of peptide cleavages. The cleaved bonds dominating IRMPD spectra may have lower dissociation energies than that of the S–S bridges. CID may be more effective that IRMPD for the peptides containing disulfide bridges, owing to different distributions of internal energy and different time frames for dissociation.

3.4. Bradykinin and Substance P

Fig. 6(a) shows the FTICR spectrum in the doubly charged Bradykinin solution (amino acid sequence: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg). The fragment ions were observed from the cleavage at Arg-Pro assigned as y_8, at Phe-Arg as b_8, at Pro-Pro as y_7/b_2 and at Ser-Pro as b_6/y_3-H_2O. Bradykinin has no acid residue and two arginine residues that are the probable protonation sites. For the single charged $[BRAD + H]^+$, the major product ions (y_8, b_8, y_7) due to cleavages at Arg-Pro, Phe-Arg and Pro-Pro are also observed. Bradykinin containing two acidic residues without a basic one has no selective fragmentation at the charge states: $z = 1$ and 2. As shown in Fig. 6(b), the lowest laser power to observe the fragment peaks of b_2/y_7 in the doubly charged $[BRAD+2H]^{2+}$ is 1.5 W. Among the dissociation channels of Arg-Pro, Pro-Pro, Ser-Pro and Phe-Arg, the peptide bond at Pro-Pro(b_2/y_7) shows the lowest energy to cleave the bonds in the protonated Bradykinin. Qualitatively similar fragmentation patters for the protonated Bradykinin have also been reported by Schneider et al. [39].

Fig. 7(a) and (b) shows the FTICR spectrum for the doubly charged Substance P (amino acid sequence: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met) and fragmentation curves. The dominant fragment ions were observed from the cleavage in the parent peptide at Leu-Met assigned as $b_{10}{}^{2+}$ and at Pro-Lys as b_2/y_9. The peptide does not contain acidic residues but two basic residues (Arg and Lys). It is noted that the most dominant peak is b_2

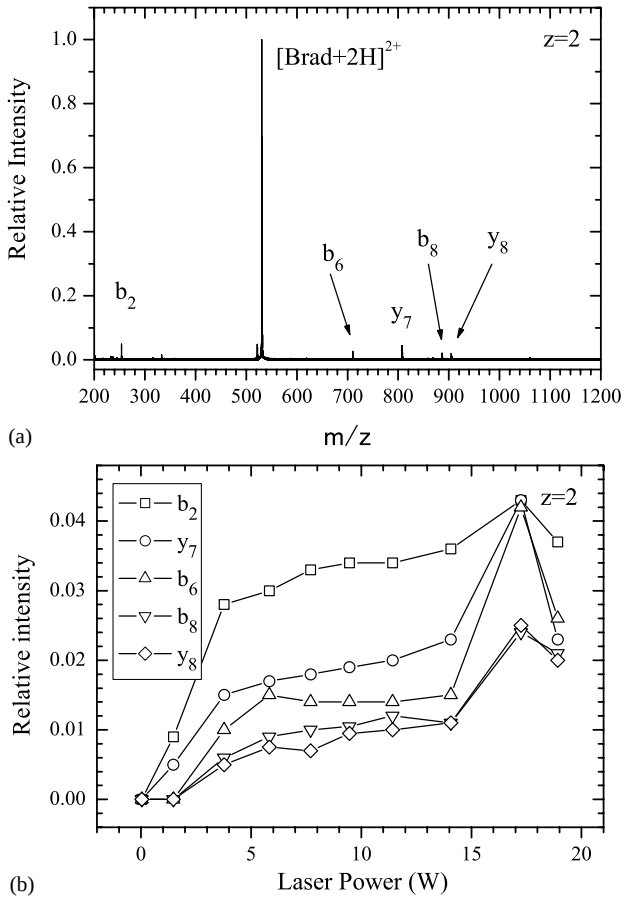

Fig. 6. (a) ESI FTICR mass spectrum of doubly charged Bradykinin at the irradiation time of 100 ms with the CO_2 laser power set at 11.3 W. (b) Dependence of fragment ions on CO_2 laser power in the range from 0 to 20 W.

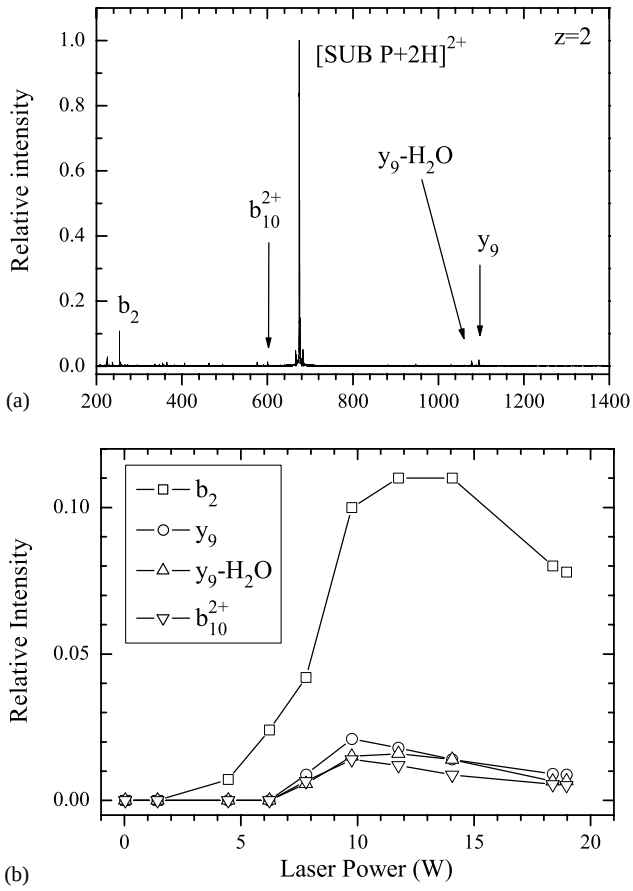

Fig. 7. (a) ESI FTICR mass spectrum of doubly charged Substance P at the irradiation time of 100 ms with the CO_2 laser power set at 10 W. (b) Dependence of fragment ions on CO_2 laser power in the range from 0 to 20 W.

arising form cleavage at Pro-Lys, not at Lys-Pro followed by proline-directed fragmentation process. The lowest laser power to observe the b_2/y_9 pair ions is 4.3 W in the doubly charged ion. In IRMPD, the selective fragmentations at the charge states were not observed.

3.5. Fragmentation analysis

Selective cleavages of secondary structure on protonated peptides have been studied to find the relationship between the number of ionizing protons and acidic/basic residues. Gu et al. have shown the selective cleavage at Asp-Xaa in a peptide containing Arg and Asp when the number of charge states does not exceed the number of Arg [4]. In our fragmentation studies of the peptides, this rule can be applied to the peptides: Angiotensin I, Angiotensin II and Urotensin II that contain an acid and a basic amino acid residue. For these singly charged peptides in IRMPD experiments, we observe the extensive fragmentation peaks caused by cleavage of Asp-Xaa (without intra-molecular proton transfer). In contrast, the peaks cleaved from Asp-Xaa disappear and the peaks arising from cleavage at Xaa-Pro are dominated for the doubly charged peptides. Table 1 summarizes the major fragment ions for IRMPD experiments at the charge states ($z = 1$ and 2). Comparing the fragment curves between the singly and doubly charged ions, the fragment ions produced from Xaa-Pro require less internal energy to initiate cleavage than those from Asp-Xaa. In case of Bradykinin and Substance P containing strong basic amino residues without an acidic residue, we do not observe charge selective fragmentation at $z = 1$ and 2 (with intra-molecular proton transfer). These results are consistent with previous studies of SID [4,13].

The general feature of the IRMPD spectra reflects an extremely low activation energy region achieved by a radiative activation/relaxation equilibrium, where some slow dissociation channels are difficult for SID and CID to access. For example, the loss of H_2O that mainly produced from singly charge state ions (e.g., [ANG II-$H_2O + H]^+$ in Fig. 1) is only observed in IRMPD, not in CID [40]. The dissociation channel is thought to be an extremely slow process occurring in the lowest energy region. This result suggests that a difference in the internal deposition rates between IRMPD and CID. Except for this, IRMPD and CID show the similar fragmentation behavior at low-energy dissociation and the difference of the produced fragment ions is observed at high collision energies in CID. Using ESI CID instruments, the fragmentation can be controlled by collision gas pressures and ion translation energy. The precise operation of the collision gas pressure was difficult and the working pressure range was quite narrow. In contrast to this, the fragmentation of IRMPD is easily controlled by changing the laser power. Thus, IRMPD can be advantageous in being more energy controllable than CID. As expected, FTICR MS IRMPD experiments show great sensitivity and selectivity to determine which amide bonds in the peptide are cleaved, and then quantitatively obtain the fragment ions at the selected charge state.

Table 1
Major product fragment ions for IRMPD

	IRMPD	
	$z = 1$	$z = 2$
Angiotensin II	y_7	b_6/y_2
Angiotensin I	y_9	b_2/y_8, b_4/y_6, y_9^{2+}, b_9^{2+}
Urotensin II	y_7	b_2/y_9^{2+}, b_{10}^{2+}
Bradykinin	y_8, b_8, y_7	y_8, b_8, b_2/y_7, y_6/b_3
Substance P	b_2	b_2/y_9^{2+}, b_{10}^{2+}

4. Conclusions

In recent years there has been significant interest in fragmentation analysis of polypeptides and proteins using MS following application of the IRMPD and CID technique. A highly specific fragmentation obtained by low-energy CID and IRMPD is useful to identify small amounts of proteins and to determine which amide bonds are cleaved at the selected charge state. This procedure can be recognized as an extremely sensitive approach to determine a single N-terminal residue, which is important to protein identification relying on protein databases.

IRMPD in FTICR MS instrument was performed for the ions of the fixed charged peptides (Angiotensin I, Angiotensin II, Urotensin II, Bradykinin, Substance P). Utilizing IRMPD, this research has determined the cleaving specificity in the protonated peptides at the selected charge states ($z = 1$ and 2). We observed the selective fragmentation peaks caused by cleavage of Asp-Xaa in singly charged Angiotensin I, Angiotensin II and Urotensin II ions that contain an acidic residue, Asp, and a basic residue, Arg or Lys. The peaks disappeared for those doubly charged peptides and the peaks arising from Xaa-Pro dominated. For Bradykinin and Substance P ions containing no acidic residues, the charge-state selective fragmentations were not observed. This work also provided the fragmentation efficiency curves to find the weakest bond of the protonated peptides by quantitatively measuring the ion abundance with ESI FTICR MS. From the curves, it was shown that the selective dissociation channel of Asp-Xaa in $[M + H]^+$ required more energy than that of Xaa-Pro in $[M + 2H]^{2+}$. The fragmentation efficiency curves for the doubly charged ions of the investigated peptides were shifted to lower energies relative to that of those singly charged ions. We are currently modeling protonated peptide and protein ions using molecular dynamics and molecular orbital methods to investigate the fragment pathway and preferential cleavage.

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