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RESEARCH ARTICLE

Non-Direct Sequence Ions in the Tandem Mass Spectrometry of Protonated Peptide Amides—an Energy-Resolved Study

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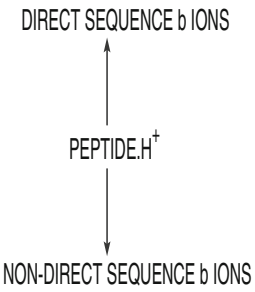

Abstract. The fragmentation reactions of the MH^+ ions of Leu-enkephalin amide and a variety of heptapeptide amides have been studied in detail as a function of collision energy using a QqToF beam type mass spectrometer. The initial fragmentation of the protonated amides involves primarily formation of b_n ions, including significant loss of NH_3 from the MH^+ ions. Further fragmentation of these b_n ions occurs following macrocyclization/ring opening leading in many cases to b_n ions with permuted sequences and, thus, to formation of non-direct sequence ions. The importance of these non-direct sequence ions increases markedly with increasing collision energy, making peptide sequence determination difficult, if not impossible, at higher collision energies.

Keywords: Peptide amides, Tandem mass spectrometry, Non-direct sequence ions

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Introduction

A common approach in peptide sequencing involves collision-induced dissociation (CID) of the protonated or multiply-protonated peptide [1–3]. In the ideal case, fragmentation occurs by cleavage of the amide bonds to give a series of y and /or b ions containing, respectively, C-terminus and N-terminus residues [4]. Usually it is these series of y and b ions that provide the sequence information. However, if these sequences of fragment ions are incomplete or unclear, difficulties may arise in determining the amino acid sequence. As a result, there has been considerable activity in studying the factors that influence the fragmentation reactions observed and the structures of the fragments formed as affected by the amino acid residues contained in the peptide.

It has been established [5, 6] that y_n ions are protonated truncated peptides although the prediction as to which y_n ions will be observed is not straightforward. Initially, the b_n ions were assumed to be substituted acylium ions [7, 8]. However, a number of studies [9–11] have shown that b_1 ions (α-aminoacylium ions) are unstable and exothermically eliminate CO to produce the respective iminium ion. By contrast, larger b_n ($n \geq 2$) ions are stable and extensively observed upon CID of protonated peptides. Extensive tandem MS, H/D exchange studies and theoretical studies of small b_n ions [12–21] have presented strong evidence that in most cases, nucleophilic attack by the adjacent carbonyl function accompanies amide bond cleavage, resulting in formation of a cyclic protonated oxazolone at the C-terminus of the b_n ion. Direct evidence for such structures has come from a number of infrared multiphoton dissociation (IRMPD) studies [22–26] of smaller b_n ions. This cyclization reaction undoubtedly accounts for the stability of b_n ions in contrast to the general instability of b_1 ions. It should be noted that in some cases, b_1 ions are observed as a result of cyclization involving a nucleophilic group in the amino acid side chain [21].

For larger b_n ($n \geq 5$) ions the situation is even more complex. Early studies [27, 28] of the fragmentation of doubly-protonated b ions containing lysyl or ornithyl residues reported the observation of non-direct sequence ions (i.e., those not expected from the known sequence of the peptides); these were rationalized in terms of cyclization/reopening reactions prior to fragmentation. More recently, a considerable number of studies [29–43] have reported the observation of more than one structure for b_5 and larger b

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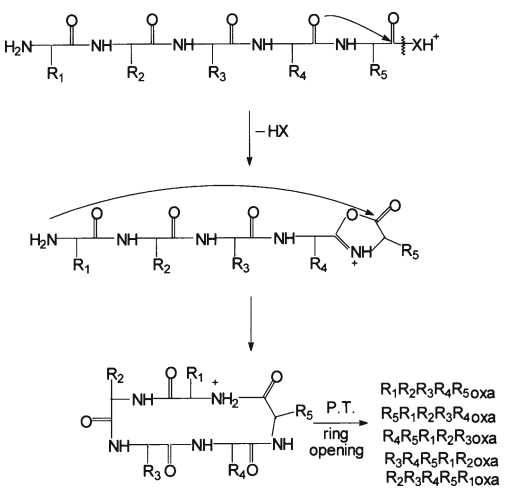
ions as well as observation of non-direct sequence ions on fragmentation of the b_n ions. These results have been rationalized [30, 33] in terms of nucleophilic attack of the N-terminal amine on the C-terminal oxazolone to form a macrocyclic isomer as illustrated in Scheme 1. Upon activation, this protonated macrocyclic isomer may reopen at different amide bonds, leading to a mixture of protonated oxazolones which, upon fragmentation, may lead to non-direct sequence ions. Recent IRMPD studies [44, 45] have shown that this macrocyclic isomer is a stable species and not a transient intermediate. Indeed, the macrocycle form is a protonated cyclic peptide. Cyclic peptides are found extensively in nature with many having physiological activity [46], but their sequencing remains difficult because of the multiple possible sites of ring opening (Scheme 1) leading to several series of fragment ions [47–51].

An important question, which has not been completely addressed, is the extent to which this sequence scrambling of b ions affects the ability to sequence unknown peptides. Siu and co-workers [52] have explored this question by examining the product ion mass spectra of 43 protonated or multiply-protonated tryptic peptides. Although non-direct b sequence ions were observed for about 35 % of the peptides studied, they concluded that these unexpected products did not affect the identification by Mascot [53]. Zubarev and co-workers [54] have studied both the low energy and higher energy dissociation of a large number of doubly-protonated tryptic peptides and concluded that, for this class of peptides, scrambling was negligible and did not interfere with sequence identification. On the other hand, Polfer and co-workers [55] analyzed a large body of data on the fragmentation of protonated tryptic peptides reported earlier [56] and concluded that there were significant signals for non-direct sequence ions particularly at low *m/z* ratios. However, it appears that these permuted sequence ions did not interfere significantly with the peptide identification in the original study [56]. Recently, Liang and co-workers [57, 58] have analyzed a large body of peptide CID spectra



Scheme 1. Macrocyclization/ring opening for b_5 ion

available in accessible data bases. They concluded that non-direct sequence ions were generally observed but with abundances less than 10 % of the base peak. They also observed that the different algorithms for peptide sequencing showed some differences in their tolerances of the inclusion of non-direct sequence ions in the data set to be analyzed. The above studies involved primarily multiply-protonated tryptic peptides, which frequently fragment primarily to form y_n ions at higher *m/z* ratios with b_n ions being observed at relatively low m/z ratios. Very recently, Bianco and co-workers [59] have reported the observation of non-direct sequence ions in the infrared multiphoton dissociation (IRMPD) of singly-protonated non-tryptic peptides although the direct sequence ions were more abundant.

Formation of non-direct or permuted sequence ions arises largely through macrocyclization of b_n ions and subsequent reopening of the macrocycle to produce oxazolones with an amino acid sequence differing from the original sequence [30, 33]. Another, probably minor, route to non-direct sequence ions may involve cyclization and reopening of a_n ions [60, 61]. Thus, the formation of non-direct sequence ions in the fragmentation of protonated peptides is most likely to occur when the initial fragmentation of the protonated or multiply-protonated peptide involves, to a significant extent, formation of b_n ions. In addition, non-direct sequence ions are more likely to be observed in beam type collisional studies where the initial b_n ions can undergo further collisional activation rather than in ion trap experiments. For example, Siu and co-workers [52] have observed more abundant non-direct sequence ions in beam-type experiments (QSTAR) than in ion trap experiments (LCQ).

Protonated peptide amides (amidated peptides) frequently show abundant b_n ions resulting, in part, from loss of NH_3 from the protonated species [62–65]. Enjalbal and co-workers [63] have reported the observation of non-direct sequence ions in the fragmentation of protonated peptide amides; indeed there are several product ion mass spectra recorded in the Supplementary Material (reported by Enjalbal et al [63]) where the non-direct sequence ions are major contributors to the overall product ion mass spectra. It should be noted that these results were obtained on a beam-type instrument. Spengler and co-workers [64] as well as Lebedev and co-workers [65] reported observation of non-direct sequence ions in the product ion mass spectra of protonated peptide amides. In both cases N-terminal amine derivatization was employed to simplify the product ion mass spectra by eliminating b_n ion macrocyclization. In the present work, we have undertaken a detailed study of the formation and prevalence of non-direct sequence ions in the product ion mass spectra of peptide amides as a function of experimental parameters in a beam type instrument. The results show that the prevalence of non-direct sequence ions is a strong function of the collision energy used making sequence determination difficult at higher collision energies.

Experimental

All experimental work was carried out using an electrospray/quadrupole/time-of-flight (QqToF) mass spectrometer (QStar XL; SCIEX, Concord, Canada). The relevant MH^+ ions were mass selected by the quadrupole analyzer Q and underwent CID in the quadrupole collision cell q with the ionic products being analyzed by the time-of-flight mass analyzer. In general experiments were carried out at a variety of collision energies as noted.

Ionization was by electrospray with the sample, at micromolar concentration, dissolved in 1:1 CH_3OH:1 % aqueous formic acid, introduced into the ion source at a flow rate of 10 μL min^{-1}. Nitrogen was used as nebulizing gas and drying gas and as collision gas in the quadrupole collision cell q.

YAGMFLV-NH_2, YAGFLVM-NH_2, YAGFLVP-NH_2, and PYAGFLV-NH_2 were obtained from GL Biochem (Shanghai, China). YPVGFLA-NH_2 was obtained from Celtek Peptides (Nashville, TN, USA) and YGGFL-NH_2 was obtained from Bachem Biosciences (King of Prussia, PA, USA). All samples were used as received.

Results and Discussion

YAGMFLV-NH_2

The product ion mass spectrum obtained for the MH^+ ion at 40 eV collision energy is shown as the top panel of Figure 1. Under these conditions, a very complex product ion mass spectrum is obtained. The product ions, which can be identified as b_n ions, are shown in the lower panel of Figure 1. The remaining ion signals in the top spectrum correspond dominantly to a_n and a_n* ions. Under these conditions, in addition to the expected b_7, b_6, b_5, b_4, b_3, and b_2 sequence ions, many non-direct sequence ions are observed. Consequently, if this was an unknown peptide it would be difficult to establish the sequence from this spectrum.

Table 1 presents the b_n ion signals as a function of collision energy from 31 eV to 42 eV collision energy. At the lowest collision energy studied, one can identify the peptide as an amide by the abundant loss of NH_3 to form the b_7 ion and obtain partial sequence information from the observation of b_6 and b_5 sequence ions. It is most likely that at this collision energy, these ions arise directly from fragmentation of the MH^+ ion rather than from further fragmentation of the b_7 ion. As one increases the collision energy further to obtain more sequence-specific b_n ions, fragmentation of the macrocyclic forms of the b_7 and b_6 ions leads to formation of non-direct sequence ions such as b_7-Y (*m/z* 619), b_7-Y-A (*m/z* 548), and b_6-Y(*m/z* 520). Thus, for example, it becomes difficult to determine whether the ion of *m/z* 570 is the true b_5 sequence ion or whether the signal at *m/z* 520 (b_6-Y) corresponds to the true b_5 sequence ion. This difficulty becomes even more pronounced with increasing collision energy and applies equally to identification of the true b_4 and b_3 sequence ions.

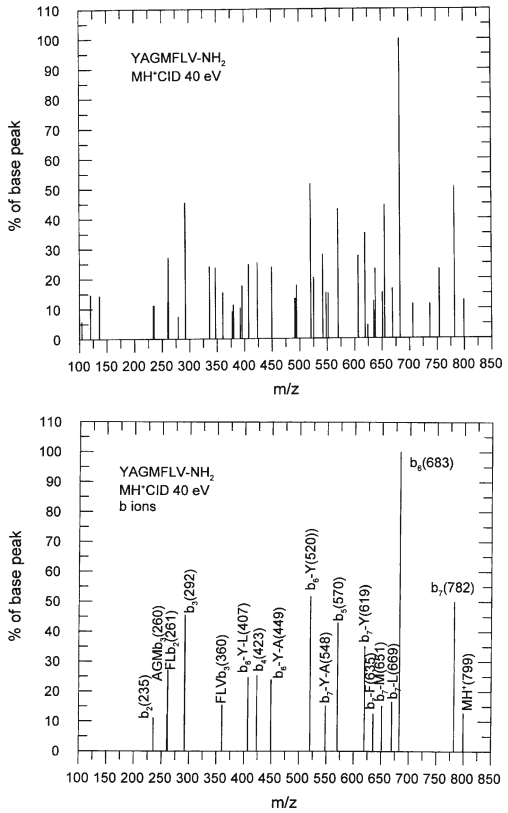


Figure 1. Product ion mass spectrum (top) and b ion distribution (bottom) for fragmentation of protonated YAGMFLV-NH_2 (40 eV collision energy)

In Figure 1 and Table 1 and in the discussion above we have identified "direct" sequence ions on the basis of the appropriate *m/z* ratio. It should be made clear that we cannot be certain of the amino acid sequence in these ions or whether the ions have an oxazolone or macrocyclic structure. Thus, the entries in Table 1 under "residues" should be taken as a statement of the residues present in the ions but not necessarily indicative of the sequence or the ion structure. This caveat also applies to the remaining results presented in the following.

YAGFLVM-NH_2

The product ion mass spectrum of the MH^+ ion at 40 eV collision energy is shown in the top panel of Figure 2 with the b_n ions identified from this plot being shown in the bottom panel of Figure 2. The variation of the b_n ion signals with collision energy is shown in the data of Table 2. The results are similar to those obtained for YAGMFLV-NH_2 presented above. The main difference is that the b_7 ion signal (loss of NH_3 from MH^+) is of lesser importance and the b_6 ion signal, resulting from loss of methionine amide from MH^+, is considerably enhanced. As a result, the non-direct sequence ions derived from macrocyclization and subsequent fragmentation of the b_7 ion are considerably reduced in importance. However, the non-direct sequence ions derived by macrocyclization/fragmentation of the b_6 ion are more pro-

Table 1. b Ions from CID of Protonated YAGMFLV-NH_2 as a Function of Collision Energy

(Intensities as % of base peak)

m/z	31 eV	33 eV	35 eV	37 eV	40 eV	42 eV	Ion	Residues
799	100	75.5	52.5	32.6	12.7	7.9	MH^+	
782	82.3	100	100	84.5	50.0	35.6	b_7	YAGMFLV
683	36.2	66.4	77.7	100	100	100	b_6	YAGMFL
669	—	—	8.8	15.6	16.5	20.2	b_7-L	VYAGMF
651	—	—	8.1	14.1	15.2	18.6	b_7-M	FLVYAG
635	—	—	6.4	11.9	12.5	16.5	b_7-F	LVYAGM
619	—	14.7	19.6	32.9	35.1	41.3	b_7-Y	AGMFLV
570	9.8	17.1	21.2	35.8	43.0	59.5	b_5	YAGMF
548	—	6.9	8.1	14.0	15.2	20.7	b_7-Y-A	GMFLV
520	—	10.5	15.6	34.7	51.7	80.0	b_6-Y	AGMFL
449	—	—	7.2	16.2	23.9	40.7	b_6-Y-A	GMFL
423	—	7.3	9.3	19.0	25.3	41.7	b_4	YAGM
407	—	—	6.4	15.0	24.7	45.8	b_6-Y-L/b_5-Y	AGMF
360	—	—	—	9.9	15.4	29.1	FLV b_3	
292	—	6.1	9.4	24.8	45.5	98.3	b_3	YAG
261	—	—	6.5	16.1	27.1	52.4	FL b_2	
260	—	—	—	6.7	12.3	27.7	AGM b_3	
235	—	—	—	6.2	11.1	25.5	b_2	YA

nounced, making the lower mass region of the product ion mass spectrum difficult to interpret. As shown by the data in Table 2, these non-direct sequence ions appear at a relatively low collision energy, at least in terms of the extent of fragmentation of the MH^+ ion.

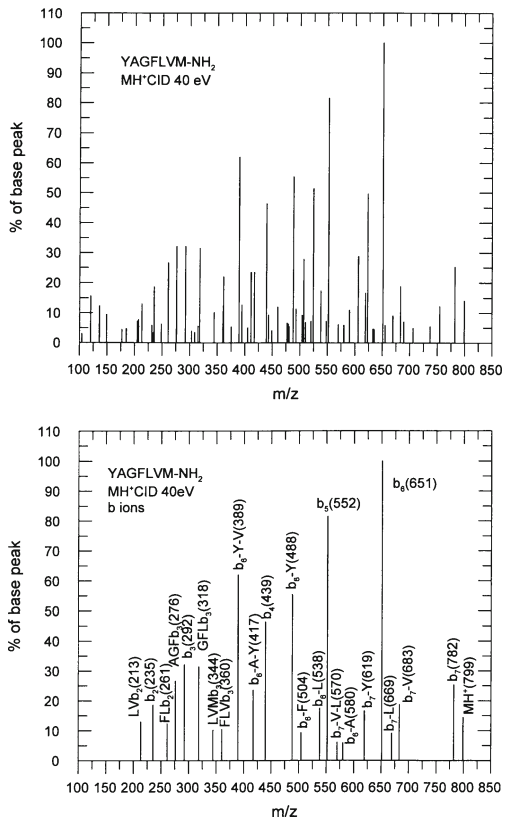


Figure 2. Product ion mass spectrum (top) and b ion distribution (bottom) for fragmentation of protonated YAGFLVM-NH_2 (40 eV collision energy)

YAGFLVP-NH_2

At low collision energies the MH^+ ion fragments almost exclusively by elimination of proline amide to give the b_6 ion. Increasing the collision energy results in minor formation of y_1 and y_2 ions and a large variety of b_n ions as shown in Figure 3 for fragmentation of MH^+ at 36 eV collision energy. Clearly the b_6 ion has undergone extensive macrocyclization and rearrangement prior to fragmentation. The distribution of b_n ions is essentially the same as one observes for fragmentation of the b_6 ion directly. Clearly, it is not possible to derive sequence information for this peptide amide from MS/MS studies of the MH^+ ion.

YPVGFLA-NH_2

The top panel of Figure 4 shows the product ion mass spectrum of the MH^+ ion at 44 eV collision energy whereas the lower panel shows the ion signals identified as b_n ions at the same collision energy. The extra remaining signals in the top panel correspond almost entirely to a_n ions resulting from loss of CO from the various b_n ions; an exception is the weak ion signal at *m/z* 602, which corresponds to the y_6 ion. Table 3 records the b_n ion signals as a function of collision energy over the range 36 to 46 eV collision energy. At the lowest collision energy studied, signals are observed for the sequence ions b_7, b_6, and b_5 ions and, surprisingly, the b_2 ion at *m/z* 261. With increasing collision energy this ion becomes the major ion in the product ion mass spectrum. Accurate mass measurements confirmed the ion as a YP (or PY) b_2 ion of unknown structure. The

Table 2. b Ions from Protonated YAGFLVM-NH_2 as Function of Collision Energy

(Ion intensities as % of base peak)

m/z	30 eV	33 eV	36 eV	40 eV	42 eV	Ion	Residues
799	100	100	43.6	14.4	6.8	MH^+	
782	34.4	58.9	46.1	25.3	17.0	b_7	YAGFLVM
683	—	10.6	13.0	18.7	19.5	b_7-V	MYAGFL
669	—	—	6.9	9.1	8.9	b_7-L	VMYAGF
651	38.9	87.7	100	100	89.3	b_6	YAGFLV
619	—	6.7	12.4	16.6	16.9	b_7-Y	AGFLVM
580	—	—	—	5.9	8.1	b_6-A	GFLVY
570	—	—	—	6.1	8.5	b_7-V-L	MYAGF
552	12.4	31.2	51.4	81.5	100	b_5	YAGFL
538	—	—	8.4	17.4	24.2	b_6-L	VYAGF
504	—	—	—	9.2	12.7	b_6-F	LVYAG
488	—	12.2	28.6	55.4	72.3	b_6-Y	AGFLV
439	—	9.3	21.7	46.3	68.5	b_4	YAGF
417	—	5.5	11.9	23.5	31.0	b_6-A-Y	GFLV
389	—	9.1	24.7	61.9	97.4	b_6-Y-V/b_5-Y	AGFL
360	—	—	—	10.4	16.4	$FLVb_3$	
344	—	—	—	10.2	16.4	$LVMb_3$	
318	—	3.5	11.4	31.4	55.5	b_6-A-Y-V	GFL
292	—	—	10.3	32.1	61.2	b_3	YAG
276	—	—	8.5	26.6	51.7	b_6-Y-V-L/b_5-Y-L	AGF
261	—	—	—	12.3	18.3	FLb_2	
235	—	—	6.0	18.6	37.2	b_2	YA
213	—	—	5.6	13.0	22.0	LVb_2	

observation of this product at low collision energies suggests the possibility that it may arise, in part, directly by fragmentation of the MH^+ ion. CID of the b_6 ion (results not shown) gave a minor yield of the *m/z* 261 product, indicating that this product does originate, at least in part, by the b ion fragmentation sequence.

The results in Figure 4 show the complete series of direct sequence ions b_7, b_6, b_5, b_4, b_3, and b_2. In addition, there are non-direct sequence ions corresponding to b_7-Y (585), b_6-Y (514), b_6-Y-L (401), and b_6-Y-L-F (254). These result from macrocyclization of the b_7 and b_6 ions with preferential reopening of the macrocycle to put the proline residue at the N-terminus of the oxazolone so-formed. An earlier study [66] of the fragmentation of b_5 ions containing proline provided qualitative evidence for the preference of the proline residue to be in the N-terminal position upon the opening of the macrocycle form.

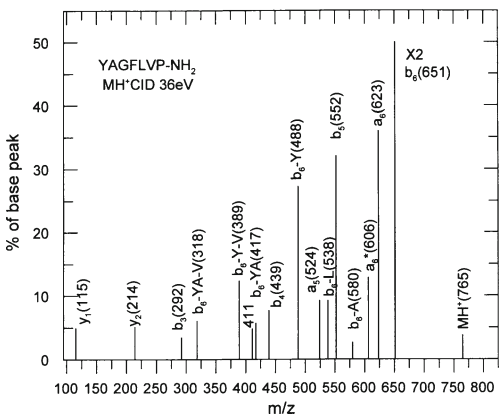


Figure 3. Product ion mass spectrum for protonated YAGFLVP-NH_2 at 36 eV collision energy

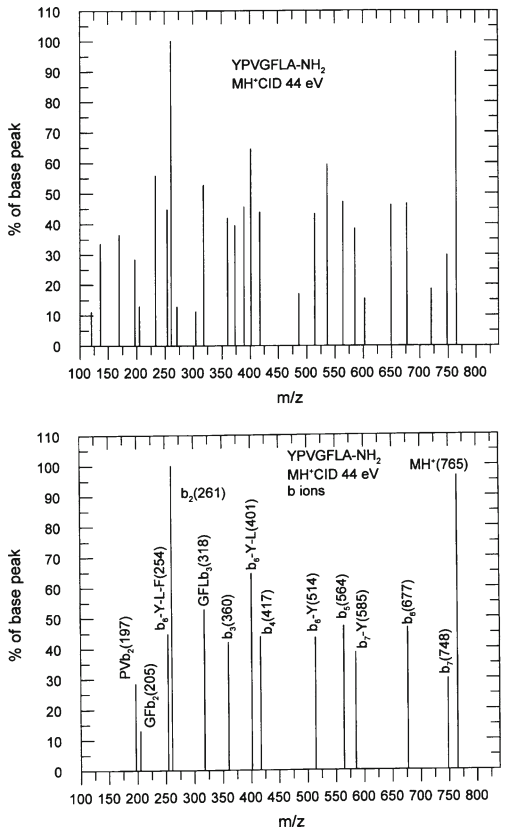


Figure 4. Product ion mass spectrum (top) and b ion distribution (bottom) for fragmentation of protonated YPVGFLA-NH_2 (44 eV collision energy)

Table 3. b Ions from Protonated YPVGFLA-NH_2

(Ion intensities as % of base peak)

m/z	36 eV	38 eV	40 eV	42 eV	44 eV	46 eV	Ion	Residues
765	100	100	100	100	96.4	43.0	MH^+	
748	11.3	14.7	19.6	24.8	29.8	15.8	b_7	YPVGFLA
677	9.7	15.3	23.0	34.5	46.6	28.6	b_6	YPVGFL
585	__	5.3	11.8	22.7	38.5	29.6	b_7-Y	PVGFLA
564	5.5	9.0	16.8	29.5	47.2	35.8	b_5	YPVGF
514	__	4.8	10.0	22.6	43.4	36.6	b_6-Y	PVGFL
417	__	5.3	10.1	22.2	43.8	42.0	b_4	YPVG
401	__	5.6	12.8	30.5	64.6	65.5	b_6-Y-L	PVGF
360	__	7.7	13.8	25.2	42.0	33.4	b_3	YPV
318	__	4.9	11.1	24.6	52.8	49.5	GFL-b_3	GFL
261	3.9	8.7	19.5	46.1	100	100	b_2	YP
254	__	4.4	7.6	19.3	44.8	49.5	b_6-Y-L-F	PVG
205	__	__	__	5.2	13.0	15.0	GF-b_2	GF
197	__	3.1	4.8	12.0	28.4	32.0	PV-b_2	PV

PYAGFLV-NH_2

The product ion mass spectrum of the MH^+ ion at 38 eV collision energy is shown in Figure 5. Not surprisingly, there is essentially no evidence for non-direct sequence ions in the spectrum. Because of the preference for the proline residue to be in the N-terminal position [66], any macrocyclic ions formed will reopen to give oxazolones with the original amino acid sequence.

YGGFL-NH_2

The product ion mass spectra of Leu-enkephalin amide at three collision energies are presented in Table 4. Although abundant direct sequence ions are observed, non-direct sequence ions are observed at *m/z* 375 (b_5-Y), *m/z* 347 (b_5-Y-CO,) and *m/z* 262 (b_5-Y-L/b_4-Y). The exact origin of the *m/z* 262 product is to some extent uncertain. Yalcin et al. [13] have observed a weak ion signal at *m/z* 262 upon the CID of the b_4 ion (*m/z* 425) of Leu-enkephalin, while Polfer and co-workers [67] have shown from IRMPD studies and hydrogen/deuterium exchange studies that the b_4 ion from Leu-enkephalin has, in part, an oxazolone structure and, in part, a macrocyclic structure. In any event, it is clear that macrocyclization of the b_5 ion and, possibly, the b_4 ion has occurred with reopening of the macrocyclic form to put the tyrosine residue at the C-terminal position.

It should be noted that the product ion mass spectrum of protonated Leu-enkephalin shows [68–70] low abundance ion signals at *m/z* 375 and 262. It has been shown [69] that in this case, these products derive, at least in part, by fragmentation of the y_4 ion (H-GGFL-OH.H^+, *m/z* 393) by elimination of H_2O (*m/z* 375) or by elimination of neutral leucine (*m/z* 262). One cannot eliminate the possibility that they may also be formed by rearrangement and fragmentation of the b_5 ion although the b_5 ion signal is much weaker for the acid than for the amide. In addition, the *m/z* 262 product may originate in part by macrocyclization/rearrangement of the b_4 ion as discussed above. It also should be noted that no signal equivalent to y_4 is observed on fragmentation of the protonated amide.

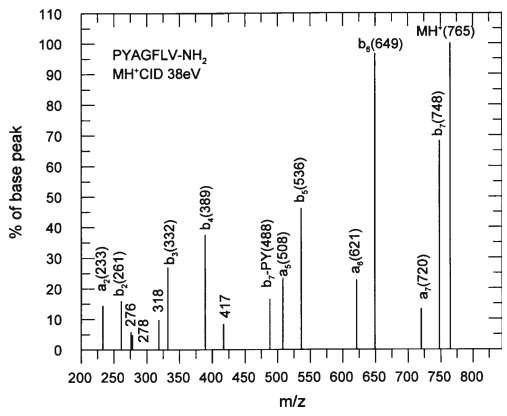


Figure 5. Product ion mass spectrum for fragmentation of protonated PYAGFLV-NH_2 at 38 eV collision energy

Table 4. Product Ion Mass Spectra of Protonated YGGFL-NH_2

(Ion intensities as % of base peak)

m/z	Ion	20 eV	24 eV	28 eV	Residues
555	MH^+	100	100	41.8	
538	b_5	59.8	92.0	75.6	YGGFL
510	a_5	14.0	57.1	100	YGGFL
493	a_5*	__	7.9	22.9	LYGGF
425	b_4	18.9	54.3	93.9	YGGF
397	a_4	__	29.7	96.5	YGGF
380	a_4*	__	__	7.8	FYGG
375	b_5-Y	5.8	29.8	65.1	GGFL
347	b_5-Y-CO	__	__	15.7	GGFL
318	?	__	__	7.8	
278	b_3	__	7.7	40.8	YGG
262	b_5-Y-L/b_4-Y	__	3.9	22.1	GGF
221	b_2	__	__	7.6	YG
136	Im_Y	__	__	10.1	Y
120	Im_F	__	__	12.0	F

Conclusions

The present energy-resolved study of the fragmentation of protonated peptide amides shows that in general, non-direct sequence ions are not observed at the lowest collision energies studied. However, at these collision energies, incomplete sequencing data are obtained. The abundance of non-direct sequence ions increases rather dramatically as the collision energy is increased, making the distinction of non-direct and nominally direct sequence ions difficult. It is not surprising that the non-direct sequence ions appear at higher collision energies since the macrocyclic forms of the direct sequence ions formed at low collision energies are stable species with a barrier for ring opening and subsequent fragmentation involving at least two bond cleavages to produce the non-direct sequence ions. The necessary energy for this reopening/fragmentation comes from increasing the collision energy in multi-collision beam-type studies. It should be noted that the usual collision energy for peptide sequencing on the QStarXL is in the range 30–40 eV, roughly the energy range used in the present study.

In general, protonated peptide amides show more pronounced formation of b_n ions than peptides terminated by a carboxyl group. In the second case there is more pronounced formation of y_n ions. This is particularly true for multiply protonated tryptic peptides, where b_n ions tend to be observed at lower *m/z* ratios. Thus, it is likely that the observation of non-direct sequence ions will be more prevalent for non-tryptic peptides and particularly for peptide amides.

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