

Effect of N-Terminal Glutamic Acid and Glutamine on Fragmentation of Peptide Ions

Bhaskar Godugu, Pedatsur Neta, Yamil Simón-Manso, and Stephen E. Stein

Chemical and Biochemical Reference Data Division, National Institute of Standards and Technology, Gaithersburg, Maryland, USA

Abstract

A prominent dissociation path for electrospray generated tryptic peptide ions is the dissociation of the peptide bond linking the second and third residues from the amino-terminus. The formation of the resulting b_2 and y_{n-2} fragments has been rationalized by specific facile mechanisms. An examination of spectral libraries shows that this path predominates in diprotonated peptides composed of 12 or fewer residues, with the notable exception of peptides containing glutamine or glutamic acid at the N-terminus. To elucidate the mechanism by which these amino acids affect peptide fragmentation, we synthesized peptides of varying size and composition and examined their MS/MS spectra as a function of collision voltage in a triple quadrupole mass spectrometer. Loss of water from N-terminal glutamic acid and glutamine is observed at a lower voltage than any other fragmentation, leading to cyclization of the terminal residue. This cyclization results in the conversion of the terminal amine group to an imide, which has a lower proton affinity. As a result, the second proton is not localized at the N-terminus but is readily transferred to various peptide bonds, leading to fragmentation at various sites near the center of the peptide. Further confirmation was obtained by examining peptides with N-terminal pyroglutamic acid and N-acetyl peptides. Peptides with N-terminal proline maintain the trend of forming b_2 and y_{n-2} because their ring contains an imine rather than imide and has greater proton affinity.

Introduction

Peptide ion fragmentation by tandem mass spectrometry has become a routine means of determining peptide sequence for the purpose of protein identification [1]. A prominent dissociation path for electrospray-generated tryptic peptide ions is the breaking of the peptide bond linking the second and third residues from the amino-terminus [2 - 4]. The formation of the resulting b_2 and y_{n-2} fragments from diprotonated peptide ions (with n amino acid residues) has been rationalized by specific facile mechanisms [2]. It has been pointed out, however, that certain peptides ions do not fragment preferentially by this route but rather fragment at peptide bonds closer to the center of the peptide [2]. A recent study of several synthetic $(Ala)_xHis$ peptide ions indicated that this fragmentation pathway diminished with increasing peptide length [4]. In the present study we attempt to distinguish between these two groups of peptides by searching for correlation between the mode of fragmentation and the amino acid sequence, mainly the amino acids at or near the N-terminus. After finding certain differences through statistical analysis of a large database of peptide MS/MS spectra, we synthesized specific peptides to study their fragmentation as a function of collision energy in an attempt to elucidate the mechanistic details. We find a significant difference in behavior when the N-terminus is either glutamine or glutamic acid as compared with all other amino acids. The reason for this difference is rapid loss of water from Q or E, leading to decreased basicity of the N-terminus and easier transfer of its added proton.

Experimental¹

The peptides were synthesized with an AAPPTEC (Louisville, Kentucky) APEX 396 synthesizer by using standard procedures. The peptides were dissolved in methanol/water (v:v = 1:1) containing 0.1% formic acid. Electrospray ionization mass spectrometry was carried out with a Micromass (Waters Corp., Milford, Massachusetts) Quattro Micro triple quadrupole instrument. First the mass spectrum was observed at different cone voltages to determine the voltage at which the protonated peptide ion peak is maximized.

¹Certain commercial equipment, instruments, or materials are identified in this document. Such identification does not imply recommendation or endorsement by the [National Institute of Standards and Technology](#), nor does it imply that the products identified are necessarily the best available for the purpose.

Then, selecting this precursor (p) ion at that cone voltage into a collision cell (with 0.21 Pa (1.6 mTorr) Ar as collision gas), the MS/MS spectrum was measured at 20 different collision voltages. The range of collision voltage spanned from near zero up to a value where little precursor ion remained. The peak intensities of all the significant fragment ion peaks were calculated as a fraction from the total ion intensity and plotted as a function of collision voltage. Spectra were acquired in ‘centroid’ mode. Typically, relative m/z values were within 0.2 of the theoretical m/z values throughout the m/z range of interest. For comparison, the spectra of several peptides were also measured with a linear ion trap instrument (LTQ, Thermo Electron Corp., Waltham, Massachusetts) at a normalized collision energy setting of 35% of the maximum.

Results and Discussion

Recent reports of principal component analysis of a mass spectral database of diprotonated peptide ions revealed two types of fragmentation behavior [2], one leading to predominant formation of b_2 and y_{n-2} fragments and the other showing minimal formation of these ions and greater fragmentation closer to the center of the peptide. A more recent study on several synthetic $(\text{Ala})_x\text{His}$ peptides showed decreased relative intensities of b_2/y_{n-2} ions with increasing peptide length [4]. To further examine the distinction between these two groups of peptides, we analyzed protonated peptide ions in six libraries of peptide mass spectra: human, yeast, *E. Coli*, mouse, *Drosophila*, and *C. Elegans*. These libraries contained 432,446 MS/MS spectra of tryptic peptide ions composed of ≤ 20 residues in charge state 1+ (12%), 2+ (65%), or 3+ (22%). Plots of different y -ion intensities as a function of peptide length (Figure 1) show the dominance of y_{n-2} ions over other y ions. For diprotonated peptides containing one basic residue (R, K, or H), y_{n-2} is very dominant for peptides with < 12 residues (Figure 1a). The effect is somewhat similar in diprotonated peptides containing two basic residues (Figure 1b). Triprotonated peptides with one basic residue show dominant y_{n-2} for the longer peptides (Figure 1c), but peptides with only 7 residues exhibit more intense y_{n-1} ion peaks. Monoprotonated peptides with one basic residue (no “mobile” protons [5, 6]) show y_{n-2} only slightly higher than the other y ions (Figure 1d).

An example of the distribution of the above statistical results is plotted in Figure 2 for the 10,658 peptide ions containing 10 residues (tryptic, diprotonated, one basic amino

acid). For each y ion, the different fractional intensities were divided into 20 bins, each differing by a factor of 2, and the number of peptide ions in each bin is plotted against bin number (expressed as percent from the maximum value). A logarithmic plot is chosen to show greater separation among the low intensity bins. It is clear from the plot that y_8 is the dominant peak in at least 70% of the spectra.

Deeper examination of the above general results shows that the tendency to produce dominant y_{n-2} ions also depends on the N-terminal residue (Figure 3). No systematic reduction of y_{n-2} was found for specific residues at the second or third position from the N-terminus (data not shown). Whereas most N-terminal residues lead to similar behavior, E and Q lead to much smaller intensities for the y_{n-2} ion peaks, but the difference decreases with increased peptide length. Since N-terminal E and Q can undergo loss of water or ammonia to form pyroglutamic acid [7, 8], we examined the importance of these losses. It has been reported that tryptic peptides with N-terminal glutamine undergo loss of either water or ammonia when singly protonated, but predominantly loss of water when doubly protonated [8]. A plot of peak intensities due to both losses in monoprotonated peptides shows the prevalence of these losses with N-terminal E, Q, and H over all other residues (Figure 4b). However, a plot for diprotonated peptides (with one “mobile” proton) shows that water loss from N-terminal Q is highly pronounced but the behavior of E falls within the range of all other residues (Figure 4a). It is likely that the predominant loss of water from N-terminal Q in diprotonated peptide ions may be the cause for the greatly diminished formation of y_{n-2} ions in such peptides. This supposition, however, does not explain the behavior of peptides with N-terminal E.

To elucidate the mechanism for the different behavior of N-terminal glutamine and glutamic acid as compared with all other amino acids, peptides were synthesized with variations in peptide length (5 to 10 residues), C-terminal K or R, and various N-terminal residues, including pyroglutamic acid and N-acetyl derivatives. Representative MS/MS spectra of diprotonated peptide ions are compared in Figure 5. LALVFAYK shows y_6 as the most intense peak and b_2 as the most intense of the b ions. The relative intensity of the y_6 ion peak is greater in the LTQ mass spectrometer (bottom spectrum) than in the Micromass instrument (top spectrum) because of differences in collision energies, but the general trends are the same. Similar trends were found when N-terminal L was replaced

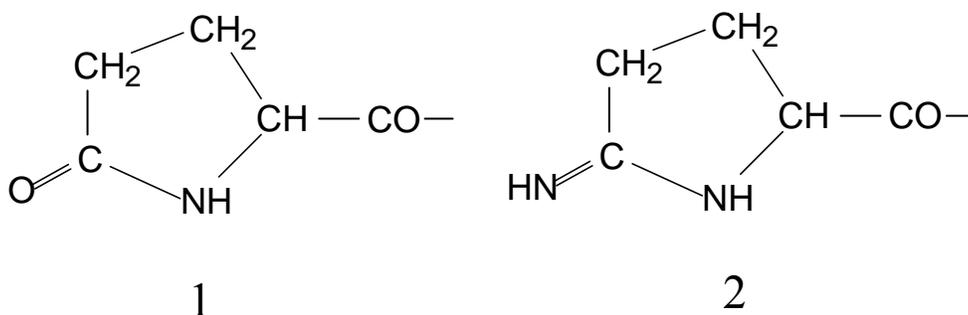
with A, N, or P (not shown). With N-terminal E, however, the most intense peak is y_4 while y_6 is much weaker in both mass spectrometers. With N-terminal Q also y_6 is much weaker than y_4 but the dominant peak is that due to loss of water from the precursor ion (p-w).

Detailed comparison of the fragmentation of these ions is shown by the collision voltage dependence of their fragment peaks (Figure 6). For diprotonated ions, MS/MS spectra were recorded at 20 collision voltages between 1 and 20 V, showing the gradual formation of each fragment with increasing voltage and its decrease at higher voltage due to further fragmentation. In Figure 6 only the range from 7 to 17 V is presented and only the most intense peaks are shown. With diprotonated LALVFAYK, the y_6 ion dominates at low collision voltage and remains dominant throughout the presented range. Similar behavior was observed when N-terminal L was replaced with A, N, or P (not shown). With N-terminal E, however, y_4 clearly dominates over y_6 and y_5 , and in addition, a peak due to water loss (p-w) is clearly evident at low collision voltage. This indicates that the diprotonated EALVFAYK ion undergoes water loss before significant peptide bond fragmentation and this water loss leads to different selectivity in peptide bond cleavage. The p-w peak is much more pronounced in QALVFAYK than in EALVFAYK and persists at higher collision voltage, i.e. requires more energy to undergo fragmentation. Similar trends for the y ions were observed with peptides containing 5 to 10 amino acid residues, with C-terminal K or R. All peptides with N-terminal E or Q undergo water loss at low collision voltage, before any peptide bond fragmentation is apparent. This water loss is followed by peptide bond fragmentation at various sites, with no preference to the b_2/y_{n-2} site, which is the “normal” site for most other peptides.

The main peaks in the MS/MS spectra of the diprotonated ions of 192 peptides are listed in their approximate order of intensity in the supplementary Table S1. Most peptides exhibit the “normal” fragmentation to b_2 and y_{n-2} ions, with varying relative intensities. In some cases, additional peaks are also intense but rarely are they more intense than the y_{n-2} or b_2 ions. In a few cases y_{n-2} is not the most intense peak, either because it is split into two peaks, singly protonated (denoted y_{n-2}) and doubly protonated (denoted y_{n-2}^{2+}), or because particular amino acid residues promote other fragmentation routes (see below). Peptides with N-terminal Q exhibit a dominant water-loss peak (p-w)

at all collision voltages. Peptides with N-terminal E exhibit a water-loss peak at low collision voltage, but this peak is small at high collision voltage. These two groups of peptides do not exhibit the “normal” b_2/y_{n-2} fragmentation pattern.

To investigate whether the deviant behavior of peptides with N-terminal E or Q is a result of water-loss from these residues, we synthesized three peptides with N-terminal pyroglutamic acid. These peptides do not undergo loss of water, but their dominant peaks are found to be same as those observed with the corresponding peptides with N-terminal glutamic acid or glutamine. These findings indicate that peptides with N-terminal E or Q do not fragment via the “normal” pathway because they preferentially undergo water loss, leading to alteration in the behavior of the resulting ions. Loss of water from E results in cyclization of the terminal NH_2 with the side-chain carboxyl group to form the pyroglutamic acid residue (structure 1) [9].



In this ring, the strongly basic NH_2 group is converted into a much less basic amide function. As a result, the second proton on the peptide is no longer localized at the N-terminus and can easily move to the various peptide bonds, leading to fragmentation at various positions. With N-terminal Q the situation is somewhat similar. Q-terminated peptides are known to undergo loss of either water or ammonia when singly protonated but predominantly loss of water when doubly protonated [8]. In the latter case the product is similar to pyroglutamic acid except with NH instead of O on the ring (structure 2). In this structure also the terminal NH_2 group is converted to a much less basic amide, leading to the different behavior. If the cause of this “abnormal” behavior of terminal E and Q is the result of conversion of the terminal basic NH_2 group into a much weaker

base, we expect to find the same effect when the terminal NH_2 is acetylated. Indeed, three acetylated peptides (Table S1) demonstrate this behavior.

From the above discussion it may be concluded that diprotonated tryptic peptide ions with 5 to 10 residues and one “mobile” proton exhibit the “normal” behavior of producing the b_2/y_{n-2} ions as the dominant peaks in their spectra, except for those peptides with N-terminal Q or E, which exhibit the “abnormal” behavior. This conclusion, however, is true for most, but not all, peptides. From the results in Table S1 (and additional results obtained in this laboratory) it appears that specific amino acid residues in specific positions can have profound effects on the spectra. First of all, proline strongly enhances fragmentation at its N-terminus. When P is in the third position (from the N-terminus), formation of y_{n-2} is greatly enhanced. Very often both y_{n-2} and y_{n-2}^{2+} are observed because P is slightly more basic than many other residues. This enhancement by P overcomes the effect of E and Q and changes the behavior of peptides N-terminated with E or Q from “abnormal” to “normal”. Peptides with P in the second position tend to form dominant peaks of y_{n-1}^{2+} ions, thus counteracting the “normal” behavior of many peptides. P at the N-terminus behaves like many other amino acid residues. Histidine, which is a stronger base than proline, also affects the fragmentation behavior in a similar manner, depending on its position within the peptide sequence.

Other effects are apparent for the aliphatic hydrophobic amino acids, most pronounced with V. In many peptides the V residue promotes fragmentation at its C-terminus; I and L appear to have similar but weaker effects. For example, the peptide EVFGYLAFK fragments predominantly to y_{n-2} and y_{n-2}^{2+} , i.e. the effect of V counteracts the effect of E and leads to “normal” fragmentation in this case, despite the presence of E terminus. In many other cases fragmentations at the C-termini of V, I, or L lead to enhanced peak intensities, sometimes counteracting the “normal” pattern. Finally, by contrast with the water loss from E and Q, it is noted that water loss from N-terminal ST or TT combinations [10], despite giving a dominant p-w peak, does not alter the dominance of b_2/y_{n-2} among the rest of the peaks. Water losses from terminal S, T, or D also have no effect of the fragmentation pattern. These water losses do not involve the terminal NH_2 group and do not modify its basicity.

To derive a quantitative measure of the “normal” and “abnormal” behavior discussed above we calculated the sum of the intensities of the b_2 , y_{n-2} , and y_{n-2}^{2+} ions as percent of the total intensities of all ions at a collision voltage $V_{1/2}$. The value of $V_{1/2}$ is the voltage at which the sum of intensities of all fragment peaks is equal to the remaining intensity of the precursor ion, i.e. when approximately half the precursor ions underwent dissociation. The reason for choosing this value rather than a constant collision voltage is that the energy required to fragment the peptide ion depends on its mass, charge, and specific amino acid basicities [11]. The percentages calculated for different peptides vary greatly but their averages show clear differences between the different groups of peptides. For 22 peptides with N-terminal E, and not containing P, the average value is approximately 4.1 %, for 3 peptides with terminal Q the value is 4.4 %, and for 3 acetylated peptides the value is 5.1 %. On the other hand, for 108 other peptides not terminated with E, Q, or acetyl group the average value is 21 %, i.e. 4 to 5 times higher, with wide variations due to peptide length and specific amino acid effects.

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Figure Captions

Figure 1. Fractions of y -ion intensities as a function of peptide length (number of amino acids) for (a) diprotonated ions containing one basic amino acid, (b) diprotonated ions containing two basic amino acids, (c) triprotonated ions containing 2 basic amino acids, and (d) monoprotated ions containing one basic amino acid. Intensities are the sums of charge states for each y -ion.

Figure 2. Distribution of peptide ions showing different fractional intensities for the different y ions. (Plot only for diprotonated tryptic peptides containing 10 residues and no additional basic residue, intensities are sums of charge states).

Figure 3. Fractional intensities of y_{n-2} ions as a function of peptide length for peptides with different N-terminal residues (intensities are sums of charge states, y_{n-2} and y_{n-2}^{2+}).

Figure 4. Fractional intensities of water/ammonia loss ions as a function of peptide length for peptides with different N-terminal residues. (a) Diprotonated peptides and (b) monoprotated peptides, all with a single basic residue. (intensities are sums of charge states)

Figure 5. MS/MS spectra of diprotonated ions of LALVFAYK, EALVFAYK, and QALVFAYK. Top spectra were recorded with the Micromass triple quadrupole mass spectrometer at collision voltages of 17, 23, and 18 V, respectively. Bottom spectra were recorded with the LTQ ion trap instrument using a collision energy setting of 35% from the maximum.

Figure 6. Peak intensity as a function of collision voltage in the MS/MS spectra of diprotonated ions of LALVFAYK, EALVFAYK, and QALVFAYK recorded with the Micromass instrument.

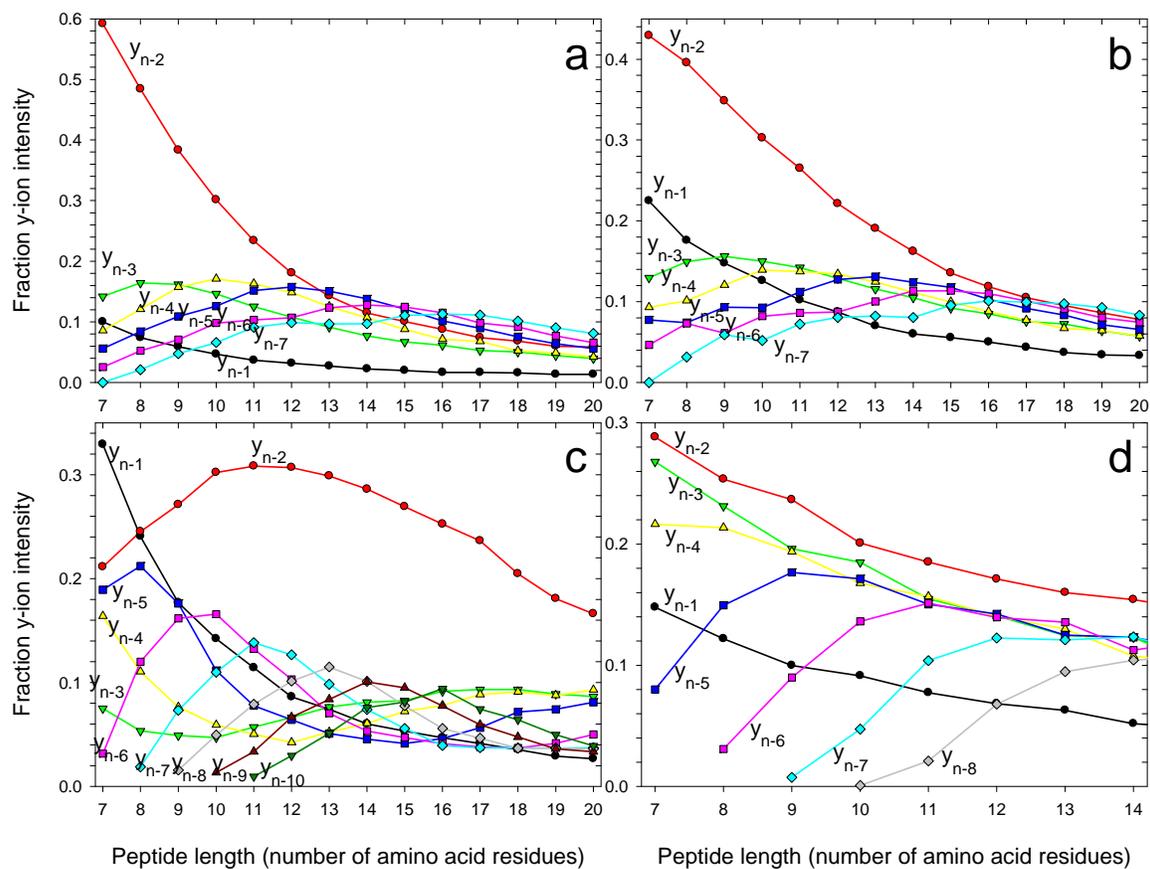


Figure 1

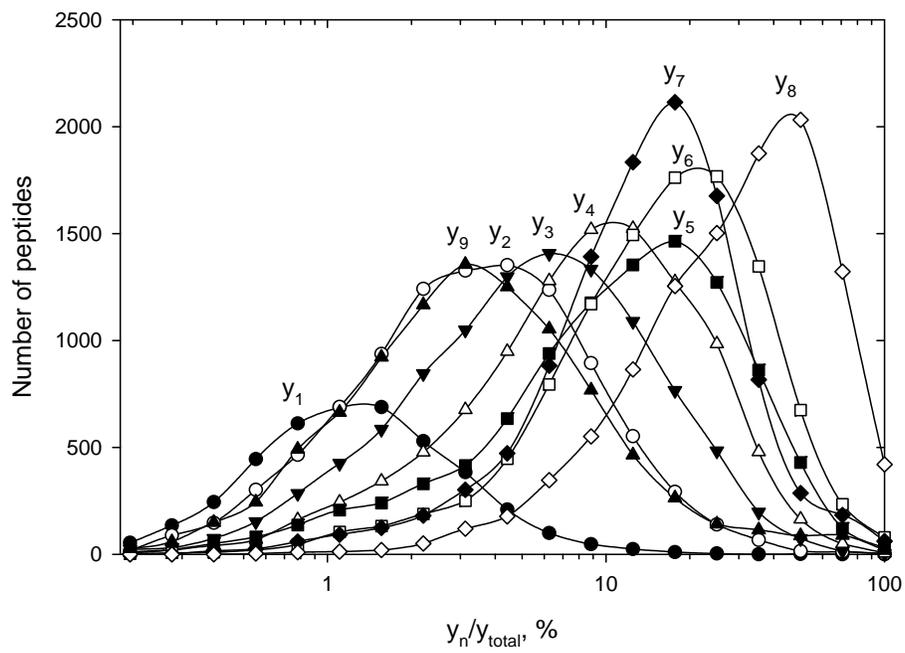


Figure 2

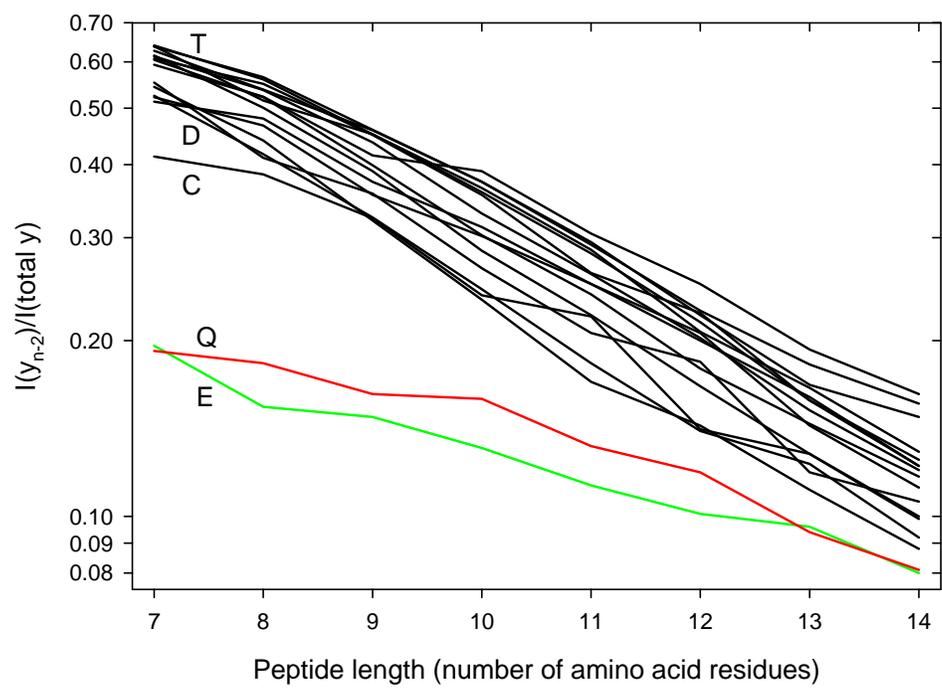


Figure 3

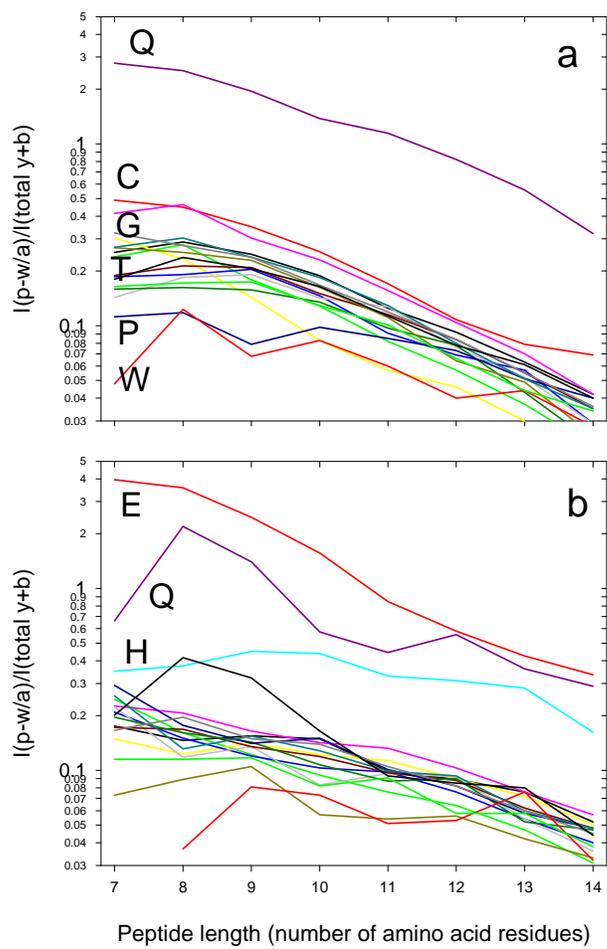


Figure 4

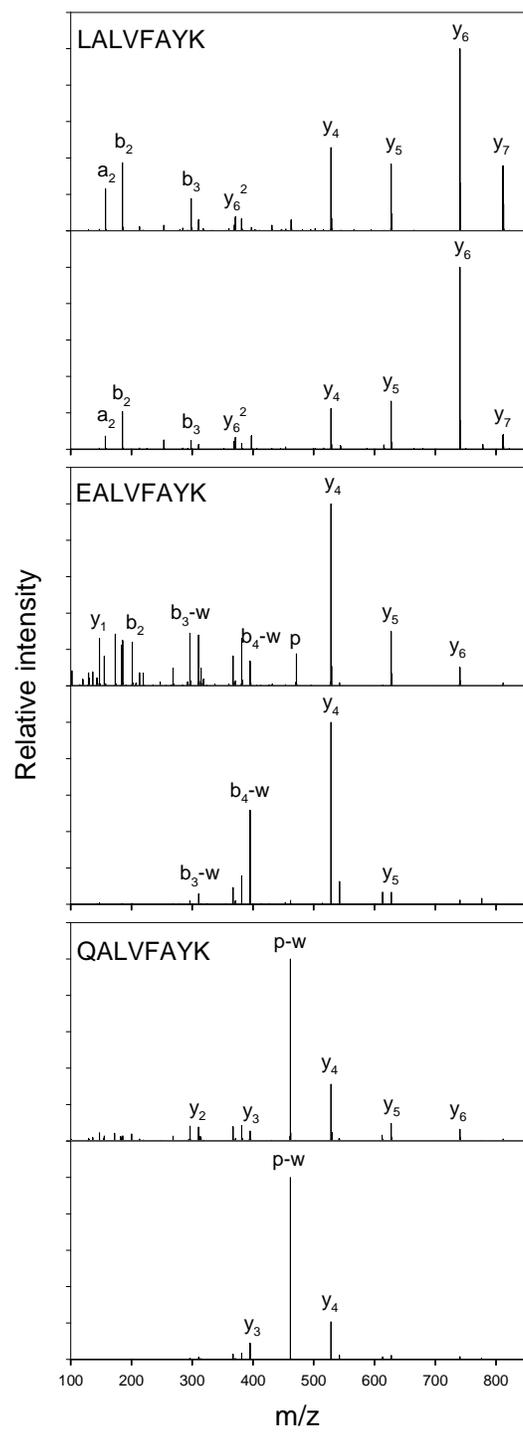


Figure 5

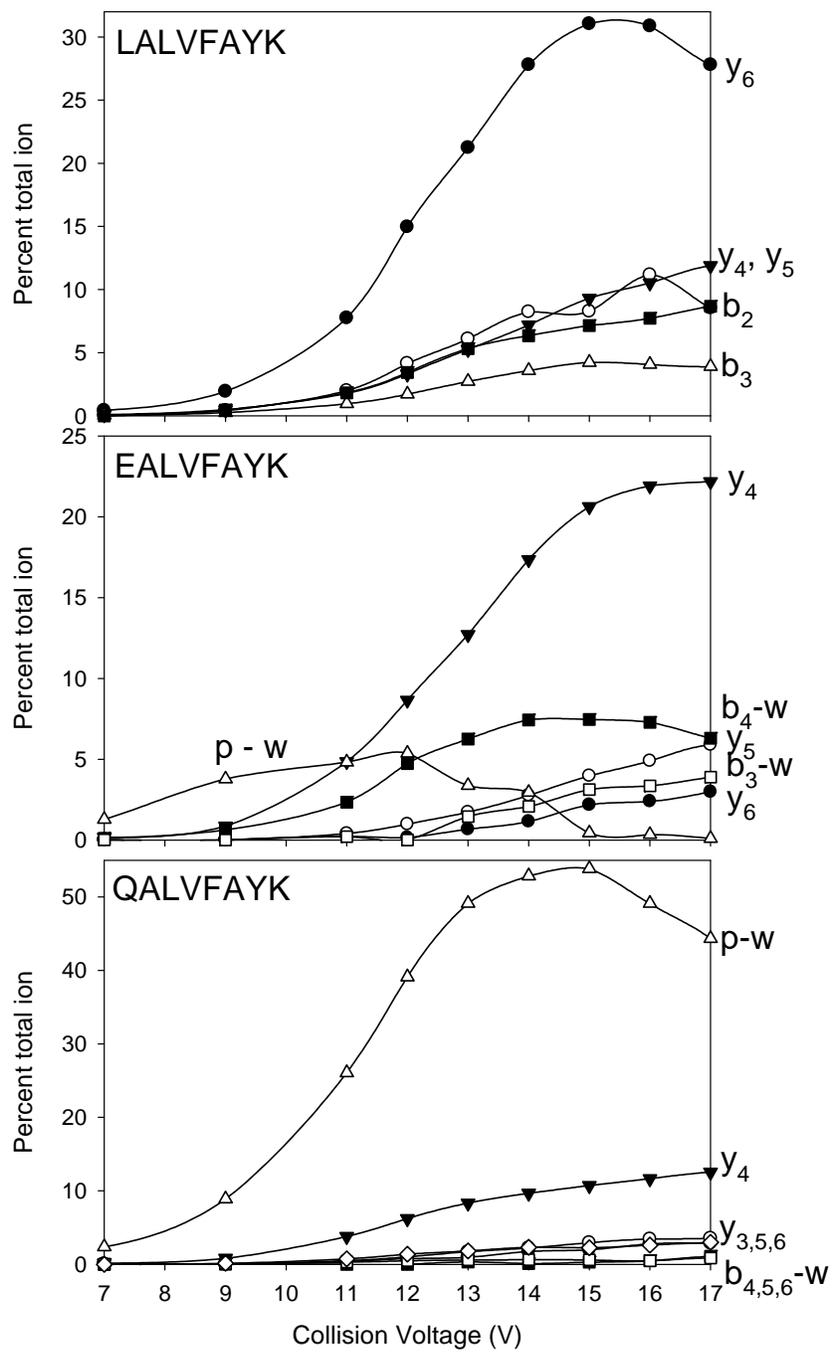


Figure 6

Table S1

sequence	m/z	main peaks in order of intensity
AAAAGTK	295.2	y ₅ , b ₂
AALVFK	324.7	b ₂ , y ₄ , y ₅ , y ₃ , y ₂
AAAAYTK	348.2	y ₅ , b ₂
AAAAAPLK	356.7	y ₆ , b ₂
AAAAAALTK	394.2	y ₇ , b ₂ , y ₆ , y ₇ ²⁺
AALVFAYK	441.8	b ₂ , y ₆ , y ₅ , y ₄ , b ₃ , y ₆ ²⁺
DALVFK	346.7	y ₄ , b ₂ , y ₃ , y ₂ , y ₄ ²⁺
DALVFAYK	463.5	y ₅ , y ₆ , y ₄ , b _{5-w} , b _{3-w}
DALVFAYR	477.8	y ₄ , b ₄ , b ₃ , y ₅ , b ₂ , y ₆
DVFGYLAFK	530.3	b ₂ , y ₇ , y ₇ ²⁺ , y ₆ , a ₂
DVFGYLAFR	544.3	y ₇ ²⁺ , y ₇ , b ₂ , y ₆
DLFVGYLAFK	586.8	y ₆ , y ₇ , y ₈ ²⁺ , y ₈ , b ₂ , b ₃
DLFVGYLAFR	600.8	y ₆ , b ₄ , y ₈ ²⁺ , b ₃ , y ₇
FFDFFR	439.6	y ₄ , b ₂
FFFFFR	455.7	y ₄ , b ₂
FFFDFFFR	586.7	y ₆ , b ₂
FLNVGLYAFR	600.3	y ₆ , y ₈ , b ₂ , b ₃
FLNVDGYAFR	601.3	y ₈ , y ₆ , b ₂ , b ₃
FLNVDAYAFR	608.3	y ₈ , y ₆ , b ₂ , b ₃
FLNVDVYAFR	622.4	y ₆ , y ₈ , b ₂ , b ₃
FLNVDTYAFR	623.4	y ₆ , y ₈ , b ₂ , b ₃
FLNVDIYAFR	629.4	y ₆ , y ₈ , b ₂ , b ₃
FLNVDLYAFR	629.4	y ₆ , y ₈ , b ₂ , b ₃
FLNVDFYAFR	646.4	y ₆ , y ₈ , b ₂ , b ₃
FLNVDYYAFR	654.4	y ₆ , y ₈ , b ₂ , b ₃
FLNVDWYAFR	665.8	y ₆ , y ₈ , b ₂ , b ₃
LLLLR	314.2	y ₃ , b ₂ , a ₂
LALVFK	345.7	y ₄ , b ₂ , y ₅ , a ₂
LLLGLLK	385.4	y ₅ , b ₂ , a ₂
LLL L A T K	386.3	y ₅ , b ₂ , a ₂
LLLLLSK	400.3	y ₅ , b ₂ , a ₂
LLLLSLK	400.3	y ₅ , b ₂ , a ₂
LLSLLLK	400.3	y ₅ , b ₂ , a ₂
LLLLLPK	405.3	y ₅ , b ₂ , a ₂
LLLLPLK	405.3	y ₅ , b ₂ , a ₂
LLLPLLK	405.3	y ₅ , b ₂ , a ₂ , y ₄ , y ₆
LLLLLTK	407.2	y ₅ , b ₂ , a ₂
LLLLTLK	407.2	y ₅ , b ₂ , a ₂
LLTLLLK	407.2	y ₅ , b ₂ , a ₂
LLLLLLK	413.3	y ₅ , b ₂ , a ₂
LLLLLNK	413.8	y ₅ , b ₂ , a ₂
LLLLNLK	413.8	y ₅ , b ₂ , a ₂
LLNLLLK	413.8	y ₅ , b ₂ , a ₂
LDLLLLK	414.4	y ₅ , y ₆ , b ₂
LLLDLLK	414.4	y ₅ , b ₂ , a ₂
LLLLLQK	420.9	y ₅ , b ₂ , a ₂

LLLLQLK	420.9	y_5, b_2, a_2
LLQLLLK	420.9	y_5, a_2, b_2, y_6
LDLLLLR	428.3	y_5, b_2
LLDLLLLR	428.3	y_5, b_2
LLLDLLR	428.3	y_5, b_2
LLLLDLR	428.3	y_5, b_2
LLLLLDR	428.3	y_5, b_2
LLLLSPLK	448.9	y_6, b_2, a_2
LLTLLLLK	457.6	y_6, b_2, a_2
LLLLPLK	461.8	y_6, b_2, a_2
LALVFAYK	462.5	y_6, y_5, y_4, b_2
LLDLLPLK	462.8	y_6, b_2
LLLDPLLK	462.8	y_6, b_2
LLLDALLR	463.8	y_6, b_2
LLLEPLLK	469.7	y_6, b_2
LLELLPLK	470.0	y_6, b_2
LLLDPLL	476.7	y_6, b_2
LALVFAYR	476.8	$b_2, y_4, y_6, y_5, b_4, b_3$
LLLEPLL	483.7	y_6, b_2
LLLDLLL	484.8	y_6, b_2
LLWLLPLK	498.3	y_6, a_2, b_2, y_6^{2+}
LLLDLFLR	501.8	y_6, b_2
LLLLPLLLK	518.3	b_2, a_2, y_7
LQLLPLLLK	525.9	y_7, b_2
LVFGYLAFK	529.3	$y_7, b_2, a_2, y_7^{2+}, y_6, b_3$
LVFGYLAFR	543.3	y_7, b_2, y_7^{2+}, y_6
LLLLDGLLLR	569.8	$b_2, y_8, y_8^{2+}, y_7, b_3, y_6$
LLLLDALLR	576.8	$b_2, y_8, y_8^{2+}, y_7, b_3, y_6$
LLLLDSLRLR	584.8	$b_2, b_3, y_8, y_8^{2+}, y_6, y_7$
LLFVGYLAFK	585.9	y_8, b_2, y_6, y_7, b_3
LLLLDPLLLR	589.8	$b_2, b_3, y_8, y_8^{2+}, y_6, y_5, y_7$
LLLLDVLLLR	590.8	$b_2, b_3, y_8, y_8^{2+}, y_6, y_7$
LLLLDTLLLR	591.8	$b_2, b_3, y_8, y_8^{2+}, y_7, y_6$
LLLLDILLR	597.8	$b_2, b_3, y_8, y_8^{2+}, y_6, y_7$
LLLLDNLLLR	598.4	$b_2, y_8^{2+}, y_8, b_3, y_7, y_6$
LLFVGYLAFR	599.9	$y_6, y_8, y_8^{2+}, b_2, y_7, b_3$
LLLLDQLLL	605.4	$b_2, y_8^{2+}, b_3, y_8, y_6, y_7$
LLLLDYLLL	622.9	$b_2, b_3, y_8, y_6, y_8^{2+}, y_7$
LLLLDWLLL	634.5	$b_2, b_3, y_8^{2+}, y_6, y_8, y_7$
LLLLLLLLLLK	639.7	$b_2, y_7, y_9, y_6, b_3, y_8$
NALVFK	346.2	y_4, b_2, y_3, a_2
NALVFAYK	463.0	$y_6, y_5, p-17, b_3, b_2, y_4$
NLLLPLLLK	518.8	b_2, y_7, a_2, y_6, b_3
NVFGYLAFK	529.8	y_7, b_2, a_2, y_6
NLFVGYLAFK	586.3	y_8, b_2, y_7, b_3, y_6
PLLLK	292.2	a_2, y_3, y_4, b_2, b_3
PLLLR	306.2	y_3, a_2, y_4, b_2
PELNGK	329.3	y_5, y_4, b_2, a_2
PALVFK	337.7	b_2, y_4, y_2, y_5, b_3
PALVFAYK	454.8	$b_2, y_6, y_7, y_4, y_5, b_3$
PFLVFAYK	492.8	y_6, b_2, a_2, y_7, y_5

PVFGYLAFK	521.3	y_7, b_2, y_6, y_8, a_2
PLFVGYLAFK	577.8	y_8, b_2, a_2, y_6, y_7
SSLLLK	330.7	$y_4, b_2, y_1, p-w, y_3, y_5$
TSLLLK	337.5	$y_4, b_2, p-w, y_5$
TLLLLK	350.6	y_4, b_2, a_2
TLLLPLLLK	512.2	b_2, y_7, a_2
VLLLLGR	392.2	b_2, y_5
VLLLLAR	399.2	b_2, y_5
VLLLLPR	412.2	b_2, y_5
VLLLLTR	414.3	b_2, y_5
VLLLLNR	420.8	b_2, y_5
VLLLLDR	421.7	b_2, y_5, y_4
VLLLLFR	437.2	b_2, y_5
VLLLLYR	445.3	b_2, y_5
VLLLLWR	456.7	$b_2, y_5, y_3, y_4, y_2, y_5^{2+}$
YYYYYR	495.6	y_4, b_2
YVPEPFTER	569.3	y_7^{2+}, y_7, b_2
EALVFR	367.0	y_2, b_4-w, y_3, b_3-w
ELLVFR	388.4	y_2, b_4-w, y_3, b_3-w
EFLVFR	405.4	y_2, b_4-w, y_3, b_3-w
EALVFK	353.4	y_2, b_4-w, y_3, b_3-w
ELLVFK	374.4	y_2, b_4-w, y_3, b_3-w
EFLVFK	391.4	y_2, b_4-w, y_3, b_3-w
ESLVFK	361.4	y_2, b_4-w, y_3, b_3-w
ETLVFK	368.4	y_2, b_4-w, y_3, b_3-w
EQLVFK	382.0	y_2, b_4-w, y_3, b_3-w
EALVFAYK	470.5	y_4, b_4-w, b_3-w, y_5
ELLVFAYK	491.5	y_4, b_4-w, b_3-w, y_5
EFLVFAYK	508.5	y_4, b_4-w, b_3-w, y_5
EFLVFAYR	522.8	y_4, b_4-w, b_3-w, y_5
EGLVFAYK	463.5	y_4, b_5-w, b_4-w, y_3
EAGVFAYK	442.5	y_4, b_4-w, y_3
EGFVYLAFK	537.3	y_5, b_4-w, y_4, b_5-w
EFVGYLAFK	537.3	$y_6, y_7^{2+}, y_4, b_5-w, b_3-w$
EVFGYLAFK	537.3	$y_7^{2+}, y_7, y_6, y_4, b_5-w, b_2$
EFVGYLAFR	551.3	$y_6, y_4, y_7^{2+}, y_7, b_5-w, b_2$
ELFVGYLAFK	593.8	$y_6, b_4-w, y_8, y_7, b_2$
ELTVGYLAFK	570.8	$y_6, y_8, y_7, b_4-w, b_2$
ELFVGYLAFR	607.8	y_6, y_7, y_8, b_2, b_3
ENINELSK	473.8	y_5, y_2, y_1
ENIPELSK	465.4	y_5, b_3-w
ELLLPLLLK	526.3	y_5, b_4-w, b_3-w
ENINEPSK	465.8	y_3, y_5, y_6, b_2
ELPVFK	366.4	$y_4^{2+}, y_2, y_4, b_4-w$
EAPVFAYK	462.5	$y_6^{2+}, b_5-w, b_4-w, y_4$
ELPVGYLAFK	568.8	$y_8^{2+}, y_8, y_7, b_2-w$
QALVFAYK	470.0	$p-w, y_4$
QVFGYLAFK	536.8	$p-w, y_7^{2+}, y_7, y_6, b_2$

QLFVGYLAFK	593.3	p-w, y_6 , y_8 , y_7 , b_2
QLLLR	321.7	p-w, y_3 , y_1 , y_2
QLLLLLR	434.9	p-w, y_3 , y_5 , y_4
QLLLLLLLR	547.9	p-w, y_5 , y_4 , y_7 , y_6 , y_3
QLLLLLLLLLR	661.4	p-w, y_6 , y_5 , y_9 , y_4 , y_3
QLLLK	307.7	p-w, y_1 , y_2 , y_3
QLLLLLK	420.8	p-w, y_3 , y_1 , y_5 , y_4
QLLLLLLLK	533.9	p-w, y_5 , y_7 , y_6 , y_4
QLLLLLLLLLK	647.1	p-w, y_7 , y_6 , b_2 , b_3
QLLLPLLLK	525.9	p-w, y_5 , y_7
QLLLPLLLR	539.8	p-w, y_5 , y_5^{2+} , y_7
E(Py)FLVFAYK	499.8	y_4 , b_4 , y_3 , b_5
E(Py)FLVFAYR	513.8	y_4 , b_4 , y_3 , y_2 , b_5 , b_3
E(Py)VFGYLAFK	528.3	y_4 , y_7^{2+} , b_5 , b_4 , y_6
Ac-LALVFAYK	483.8	$b_1(\text{Ac-L})$, y_5 , y_7^{2+} , b_3 , b_2 , y_7 , y_6 , y_4
Ac-PALVFAYK	475.8	$b_1(\text{Ac-P})$, y_5 , b_2 , b_3 , y_7 , y_6 , y_4
Ac-EALVFAYK	491.8	$b_1(\text{Ac-E})$, y_7^{2+} , y_5 , y_7 , b_2 , b_4 , y_4
STLLLK	337.5	p-w, y_4 , b_2
TLLK	288.2	p-w, y_3 , b_2
TLLLK	344.7	p-w, y_4 , b_2
TPLLLK	393.1	p-w, y_5 , b_2
TLLLLR	415.2	p-w, y_5 , b_2
TLLLLLK	401.3	p-w, y_5 , b_2
TLLLLLLK	457.8	p-w, y_6 , b_2
TLLLLLLLK	514.0	p-w, y_7 , b_2 , y_6 , y_5 , y_7^{2+}
TLLLPLLLK	562.6	p-w, y_8 , y_6 , y_7 , b_2 , y_8^{2+} , y_5
TLLLLLLLK	570.6	p-w, b_2 , y_8 , y_6 , y_7 , y_5 , y_8^{2+}
TLLLLLLLLK	627.2	b_2 , y_7 , y_6 , p-w, y_9 , y_8 , y_4 , y_5
LLPLLLK	405.5	y_5 , b_2 , a_2 , y_5^{2+}
AAPAAAK	300.2	y_5^{2+} , y_5 , b_2
APAAAK	300.2	y_6^{2+} , y_5 , b_2 , y_6
CLLLPLLLK	513.4	y_7 , y_5 , y_6 , y_7^{2+} , b_3 , b_2
FFPFFFK	490.0	y_5 , b_2 , y_5^{2+} , a_2
FPFFFK	490.0	y_6^{2+} , y_5 , y_6
LPVEPFTER	544.3	y_8^{2+} , y_5 , b_2 , y_7 , y_6
YPLLPFTLR	560.3	y_5 , y_8^{2+} , b_3 , y_7 , y_6
YPVEPFTER	569.3	y_5 , y_8^{2+} , y_7 , y_6 , b_2
YPVEPFTLR	561.3	y_8^{2+} , y_5 , y_7 , y_6 , b_2
YPVLPFTLR	561.3	y_5 , y_8^{2+} , y_6 , y_7 , b_2 , b_3
VHLLLLLR	488.8	b_2 , y_7^{2+} , y_6 , b_3 , y_5
VLHLLLLR	488.8	y_6^{2+} , b_3 , y_5 , y_7^{2+} , y_4
VLLHLLLLR	488.8	y_5^{2+} , y_6^{2+} , b_2 , y_5 , y_4 , y_7^{2+}
VLLLHLLR	488.8	b_2 , y_5^{2+} , y_6^{2+} , y_4 , y_6 , y_4^{2+} , y_7^{2+} , y_5
VLLLLHLR	488.8	b_2 , y_6^{2+} , y_6 , y_5^{2+} , y_5
VLLLLHLR	488.8	b_2 , y_5^{2+} , y_6 , y_6^{2+} , y_4 , y_5
LLLDHLLR	496.8	y_6 , y_6^{2+} , b_2

HLLLPLLLK 530.3 b_2, y_7, a_2