The Strange Case of “Picket Fence” Peaks: A study in the Complexity of MS/MS Spectra of Protein Ions

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ABSTRACT

Purpose: Explain the series of 1 Th (Da/elementary charge) spaced *m/z* peaks often observed in the MS/MS spectra of large polypeptides such as intact proteins. Generally such “picket fence” *m/z* peak series have been assumed to be from some unknown interfering singly charged background species. These “picket fence” m/z peaks interfere with automated *m/z*-to-mass conversion and sequence determination.

Methods: Large polypeptide standards were subjected to ESI MS/MS using multiple dissociation methods. Ion-ion proton transfer charge state reduction reaction (PTCR) subsequent to ion dissociation is used to indicate the actual charge states of the ions that produce the “picket fence” m/z peak series. Analytical theory and in silico dissociation and analysis of the dependence of polypeptide sequence fragment mass defects on fragment mass for analyzed protein are used to explain the origin of the “picket fence” *m/z* peaks.

Results: The “picket fence” *m/z* peaks are determined to be the product of constructive interference of overlapping isotopic *m/z* peak envelopes of relatively long/highly charged dissociation product ions. Such constructive interferences only will be observed for large polypeptides with amino acid sequences with relatively uniform distributions of constituent amino acid residues.

INTRODUCTION

MS/MS spectra of multiply charged proteins are typically composed of *m/z* peaks representing hundreds of different product ion species. If the degree of dissociation is such that relatively long product ions are conserved, most of the product ion *m/z* peaks will be concentrated in the neighborhood of the precursor *m/z*. This leads to many overlapping isotopic peak envelopes. Here we show that, for many protein precursor species, the dense overlap of product ion isotopic *m/z* peak envelopes results in the observation of a series of artifact *m/z* peaks with spacing of 1 Th (Da/elementary charge) that prior to this work have generally assumed to be produced by some sort of low charge state chemical background interference and ignored. These “picket fence” *m/z* peaks frustrate m/z-to-mass “deconvolution” programs leading to missed sequence ion assignments.

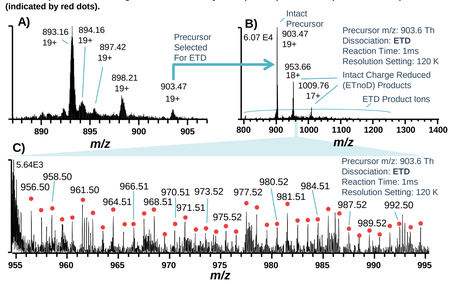
MATERIALS AND METHODS

Apomyoglobin and Carbonic Anhydrase protein standards (MilliporeSigma, St. Louis, Mo.) were infused (0.1 % Formic acid, 50% Methanol, 50 % Water solution) into a the ESI source of a Thermo Scientific™ Orbitrap Fusion ™ Lumos ™ Tribrid ™ mass spectrometer (Q-Orbitrap-q-QLT) with Electron Transfer Dissociation (ETD, Fluoranthene reagent) and UVPD (213 nm) capabilities and analyzed via MS, MS^2, and MS^3. The instrument’s ETD reagent inlet was modified to allow introduction of a second reagent compound, Perfluoroperhydrophenanthrene, (MilliporeSigma, St. Louis Mo.). which produces a reagent ion *m/z* of 624 Th. This reagent ion reacts with multiply protonated polypeptides exclusively via ion-ion proton transfer charge reduction, PTCR (also referred to in the literature as PTR and IIPT). The instrument’s control software was modified to enable PTCR as an activation type for MS^n experiments.

SILu™Lite mAb standard (MilliporeSigma, St. Louis Mo.) was digested with the IdeS enzyme and reduced (10 mM TCEP) to generate LC, Fd, and Fc/2 subunit polypeptides. These mAb subunits were on line separated via nLC - an Agilent 1100 pump (Agilent Tech. Santa Clara, CA) with a homemade nLC column (360 µm OD,75 µm ID, 100 mm long) packed with PLRP-S material (Agilent Technologies, Santa Clara, CA). 100 nl/min flow, Solvent A: 0.3% Formic acid and water, and Solvent B: 0.3% Formic Acid, 72% Acetonitrile, 28% Isopropanol, 10% Water. The LC eluent was delivered to the nESI source of a Thermo Scientific™ Orbitrap Fusion ™ Tribrid ™ mass spectrometer for *m/z* analysis. The SILu Lite Fd subunit HCD MS^2 spectra were acquired with stepped normalized collision energy (21%, 24%, 27 %).

Numerical modeling of single and superposed uniform intensity m/z combs (half cosine peak shapes) corresponding to series of integer masses at specific charge states (zero mass charge carriers) was performed in Mathematica (Wolfram Research, Champaign, IL). Calculations of neutral mono-isotopic masses for all c- and z- type fragments for Apomyoglobin, Carbonic Anhydrase and SILu Lite Fd subunit were done with a for purpose modified version of the Orbitrap Fusion Lumos instrument control software (Lua language). Plotting and fitting of neutral fragment mass defects versus fragment neutral mass numbers was done in Excel (Microsoft, Redmond WA).

Figure 1. A) Detail of MS^3 spectrum of Apomyoglobin showing a minor proteoform *m/z* selected for ETD. B) ETD MS^2 product ion spectrum of the selected Apomyoglobin precursor. C) Detail of a portion of the Myoglobin ETD MS^2 spectrum of showing a series of nominally 1 Th spaced “picket fence” product ion *m/z* peaks (indicated by red dots).

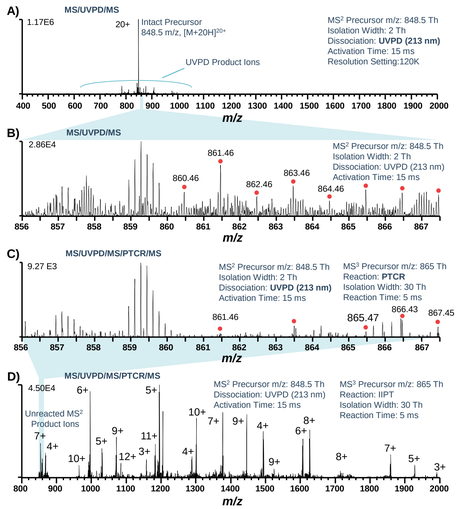


RESULTS AND DISCUSSION

A typical example of the observation of series of nominally 1 Th spaced “picket fence” product ion peaks is illustrated through the results of the pair of experiments depicted in Figure 1. A minor proteoform of Apomyoglobin was observed in the Orbitrap MS^3 spectrum of the Apomyoglobin standard (Figure 1 A). The 19+ charge state of this species was *m/z* selected with the quadrupole filter and subjected to a reaction with ETD reagent ions in the high pressure cell of the linear quadrupole ion trap for 1 ms. Such a short ion-ion reaction period permitted only a couple of generations of ion-ion reactions to occur. This is indicated by the observation of the large unreacted precursor *m/z* peaks and observation of only of the first and second generation intact charge reduced (ETnoD) product ion *m/z* peak envelopes in the Orbitrap ETD MS^2 spectrum (Figure 1B). Since the ion-ion reaction rate constants are proportional to the square of the analyte ion’s charge state, the observations of these species indicates that the population of dissociation product ions should be rich in large – more than half the mass of the intact precursor - and relatively highly charged sequence ions. The isotopic *m/z* peak envelopes corresponding to large sequence ions should be concentrated in the neighborhood of the m/z of the first and second intact charge reduced species. The distribution of the dissociation product ions in the MS^2 spectrum in Figure 1B matches this expectation. A zoomed-in view of a 40 Th portion of the ETD MS^2 spectrum in the *m/z* range just above the *m/z* range where the isotopic m/z peak envelope corresponding to first generation intact charge reduced product ions is located (Figure 1 C) exhibits an extended series of “picket fence” *m/z* peaks.

In a second pair of experiments, the 20+ charge state of the principal proteoform observed in the Apomyoglobin standard MS^1 spectrum was *m/z* selected by the quadrupole filter and subjected to a beam of 213nm photons for 15 ms in the low pressure cell of the linear quadrupole ion trap in order to effect ultraviolet photo-dissociation, UVPD. In the resulting Orbitrap UVPD MS^2 spectrum, Figure 2A, precursor m/z peak envelope is the most intense feature of the spectrum, and the product ions signal appears concentrated in the neighborhood of the precursor *m/z* indicating that the product ion population should be rich in long highly charged sequence ions. A zoomed in view of a 11.5 Th portion of the UVD MS^2 spectrum just above the precursor *m/z* (Figure 2B) exhibits a series of “picket fence” m/z peaks. However, when in an MS^3 experiment, this range of UVPD product ions is moved into the high pressure QLT, *m/z* isolated (30 Th width), and subjected to reaction with PTCR reagent ions for a mere 5 ms, the “picket fence” ions are substantially depleted (Figure 2C). In this short of a reaction period, low charge state ions (1+, 2+ and 3+) should be negligibly depleted. The corresponding full Orbitrap MS^3 spectrum (Figure 2D) shows an abundance of different charge reduced UVPD product ions which had charge states prior to PTCR as high as 16+.

Figure 2. MS^2 and MS^3 spectra of Apomyoglobin: A) Orbitrap UVPD MS^2 product ion spectrum of the selected Myoglobin precursor (primary proteoform). B) Detail of a section of the Myoglobin UVPD MS^2 product ion spectrum near the precursor *m/z* exhibiting the nominally 1 Th spaced “picket fence” product ion *m/z* peaks (indicated with red dots). C) Detail from of the same section of the UVPD product ion m/z range after reaction for 5 ms with PTCR reagent ions. The *m/z* range shown was within a 30Th *m/z* window of UVPD product ions isolated prior to the PTCR reaction. D) The complete Orbitrap MS/UVPD/MS/PTCR/MS spectrum showing an abundance of charge reduced product ions from the m/z isolated UVPD product ions.

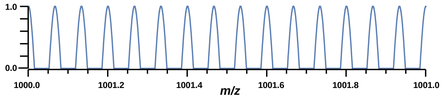


RESULTS AND DISCUSSION (CONTINUED)

The experimental results illustrated by Figures 2C and 2D clearly indicate that the product ions that are responsible for producing “picket fence” *m/z* peak series are highly charged and that the regions of product ion *m/z* spectra where these “picket fence” m/z peaks are observed are regions with a high density of overlapping isotopic *m/z* peak envelopes from highly charged product ion species.

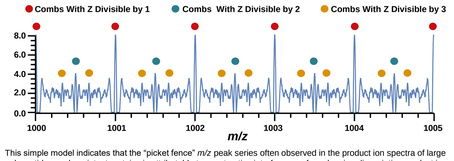
To understand how a high density of overlapping isotopic *m/z* peak envelopes could produce “picket fence” *m/z* peak series, we utilized a simple model. In this simple model, all masses are integer (there is no mass defect) and charge carriers have zero mass. Further we consider combs of uniform intensity *m/z* peaks rather than the Gaussian like profile of actual isotopic *m/z* peak envelopes and gave the individual *m/z* peaks half cosine profiles. In this model, species having masses in the neighborhood of 15000 Da when charged to 15+ produce a comb of *m/z* peaks as depicted in Figure 3.

Figure 3. Simulated comb of unit intensity *m/z* peaks (half cosine profile) having separation corresponding to a sequence of masses separated by exactly 1 Da with a 15+ charge state.



A region of a product ion *m/z* spectrum with an abundance of relatively large product ions of various high charge states would therefore be modeled simply as the superposition of *m/z* peak combs corresponding to multiple charge states. Figure 4 shows the result of such a calculation. It indicates that all *m/z* peak combs contribute via constructive reinforcement to peaks at integer *m/z* values. Even charge state *m/z* peak combs contributed via constructive reinforcement produce a series of less intense peaks at all integer *m/z* values + ½ Th. Further, *m/z* peak combs with charge states that are integer multiples of 3 yield via constructive reinforcement peaks at all integer *m/z* values ± 1/3 Th.

Figure 4. Simulated aggregate *m/z* spectrum from the superposition of combs of unit intensity *m/z* peaks (half cosine profile) having separations corresponding to sequences of masses separated by exactly 1 Da. The superposed *m/z* peak combs correspond to charge states of 8+,9+,10+,11+,12+,13+,14+, and 15+. Peaks in the aggregate *m/z* spectrum produced by constructive interference of *m/z* peak combs with charge states (Z) divisible by 1, 2 and 3 are indicated.



This simple model indicates that the “picket fence” *m/z* peak series often observed in the product ion spectra of large polypeptides such as intact proteins is attributable to constructive interference of overlapping dissociation product ion isotopic *m/z* peak envelopes. However, the masses (in Da) of the isotopes of the elements that comprise polypeptide sequence fragments (H, C, N, O, and S) are not integers. The monoisotopic mass of any given neutral polypeptide sequence fragment serves as the anchor for its entire associated set of isotopic neutral masses that when multiply protonated produce isotopic *m/z* peak envelopes. To understand how such constructive interferences may persist in a universe where mass defect exist, let us consider how the mass defects of the monoisotopic masses of neutral sequence fragments change with their size. For purposes of this analysis, it is advantageous to define for a given neutral sequence fragment, identified by an index *i*, its mass defect, D_i, as the difference between its monoisotopic neutral accurate mass, M_i, and its monoisotopic mass number N_i (mass calculated with integer elemental masses: C 12 Da, N = 14 Da ,O = 16 Da).

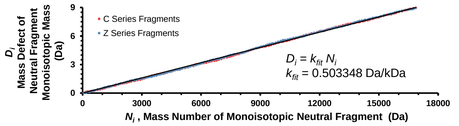
$$M_i = N_i + D_i$$

By referencing mass defect to mass number rather than the next lower integer mass, the mass defects, D_i, for the neutral monoisotopic masses of sequence fragments of any polypeptide can be well estimated as being proportional to the fragment’s mass number, N_i

$$D_i = k_{fit} N_i$$

The constant of proportionality for a given polypeptide with a known amino acid residue sequence, k_{fit} , may be determined by a least squares fit of the calculated D_i, N_i pairs for an entire complimentary series of sequence fragments as is demonstrated for Apomyoglobin in Figure 5.

Figure 5. The mass defect, D_i, relative to neutral monoisotopic mass number, as a function of the mass number for all C-type and Z-type monoisotopic sequence fragments of Apomyoglobin.



RESULTS AND DISCUSSION (CONTINUED)

How well sequence fragment neutral monoisotopic masses conform to proportionality between mass defect and mass number depends upon the amino acid sequence of the polypeptide. For every sequence fragment there is a residual fit error value, ε_i such that:

$$D_i = k_{fit} N_i + \varepsilon_i \quad .$$

Plots such as those in Figure 6 showing the variation and size of the fit error values, ε_i, as a function of sequence fragment neutral mass number N_i are indicative of the conformity to proportionality D_i with N_i for the particular polypeptide. Note that the fitted proportionality constant, k_{fit}, differ slightly for the three polypeptides. This is likely due to variations in amino acid composition and sequence order for the different polypeptides. Refer to Table 1 to determine how the different amino acid residues contribute fragment neutral mass defects. Such plots also appear to provide some guidance to whether “picket fence” m/z peaks may be observed in the MS^2 spectra of the polypeptide. Figure 6B shows that Carbonic Anhydrase has much higher D_i to N_i proportionality fit errors, ε_i, than are shown for Apomyoglobin in Figure 6A and SILu Lite mAb Fd subunit in Figure 6C. We have failed to observe “picket fence” *m/z* peak series in any MS^2 spectra using Apomyoglobin (Figures 1C and 2B) and SILu Lite mAb Fd subunit (Figure 7) precursor ions.

Using the expression for the mass defect of the sequence fragment neutral monoisotopic mass given above, we can derive the following expressions for the sequence fragment neutral monoisotopic mass, M_i.

$$M_i = N_i + k_{fit} N_i + \varepsilon_i = (1 + k_{fit}) N_i + \varepsilon_i$$

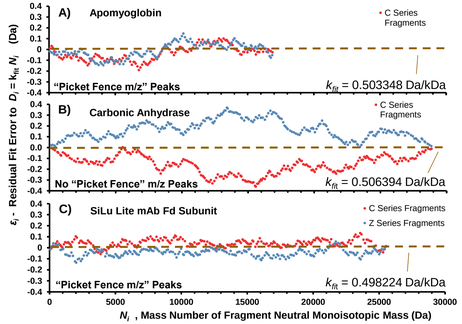
From this expression we can derive an expression for the *m/z* of the multiply protonated monoisotopic ion for any sequence fragment (index *i*) with charge state Z_i.

$$\lceil m/z \rceil_i = \frac{(1 + k_{fit}) N_i + \varepsilon_i + Z_i \lceil m/z \rceil_{Proton}}{Z_i} = (1 + k_{fit}) \frac{N_i}{Z_i} + \lceil m/z \rceil_{Proton} + \frac{\varepsilon_i}{Z_i}$$

The fitted quantity k_{fit} is very near 0.0005 Da/Da for any of the polypeptides in Figure 6. For different sequence fragments with monoisotopic masses that, when ionized, have N_i/Z_i ratios that are sufficiently close (within about 1Th) of each other so that their associated isotopic *m/z* peak envelopes will likely overlap, the quantity $(1 + k_{fit})$ simply introduces a collective shift (to within about 0.0005 Th for $N_i/Z_i = 1000$ Th) in *m/z* position of the monoisotopic *m/z* peaks. For $N_i/Z_i = 1000$ Th, the magnitude of this common m/z shift is about 0.50 Th. This locally common displacement is in addition to the “global” collective shift of 1.00729 Th in monoisotopic *m/z* peak positions introduced by the proton *m/z* term. However, the ε_i / Z_i term will introduce shifts in the relative positions of monoisotopic *m/z* peaks of the different sequence fragments which could prevent constructive reinforcement of *m/z* peaks overlapping isotopic envelopes. The spacing between isotopic *m/z* peaks (approximately 1 Da/Z) is negligibly altered by these considerations, so the overlapping isotopic envelopes will behave like the superposed combs of *m/z* peaks if the relative shifts in monoisotopic *m/z* peak positions introduced by the ε_i / Z_i term are small compared to *m/z* peak widths. Note that the higher charge state the smaller the relative shift in monoisotopic m/z peak position for a given ε_i value. So, larger sequence fragments will therefore have smaller relative *m/z* displacements.

For Carbonic Anhydrase, which has values for ε_i of range of ±0.20 Da for N_i values in the range of 10,000-15,000 Da, the ε_i / Z_i term would lead to relative m/z shifts of the monoisotopic m/z peaks of about ±0.017 Th for monoisotopic *m/z* peaks in the neighborhood of 1000 Th. These displacements are approaching the *m/z* peak widths for that *m/z* at the 120 K resolution setting of the Orbitrap analyzer. For Apomyoglobin and For SILu Lite mAb Fd subunit the values for ε_i over the same range of N_i values are respectively about 1/3 and 1/5 of those for Carbonic Anhydrase. Therefore, of the three examined polypeptides, SILu Lite mAb Fd subunit would be expected to exhibit constructive interferences that would most closely match what would be predicted from Figure 5. Indeed the portion of a HCD MS^2 of the 18+ charge state of SILu Lite mAb Fd subunit shown in Figure 7 clearly exhibits both the 1 Th (induced by all charge products states) and 0.5 Th (induced by even charge state products) interval “picket fence” m/z peaks predicted in Figure 4.

Figure 6. Residual error in a least squares proportional fit of the mass defect, D_i (relative to neutral monoisotopic mass number, N_i) to N_i the for all C-type and Z-type fragments for A) Apomyoglobin, B) Carbonic Anhydrase, C) SiLu Lite mAb Fd Subunit. The fitted constant of proportionality, k_{fit}, is given for each polypeptide.

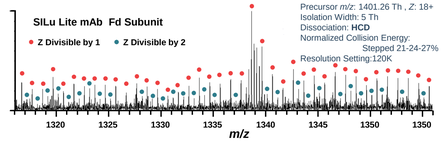


RESULTS AND DISCUSSION (CONTINUED)

Table 1. A list of the amino acid residues including their accurate masses (M), mass numbers, mass defects, and relative mass defect (*D/N*). This list is ordered in increasing relative mass defect.

AA Code	Amino Acid Residue	Residue Formula	*M* Monoisotopic Mass (Da)	*N* Mass Number (Da)	*D* Mass Defect (Da)	*D/N* (Da/kDa)
C	Cysteine	C_3H_5NOS	103.009185	103	0.009185	0.089
D	Aspartic acid	$C_4H_5NO_3$	115.026943	115	0.026943	0.234
M	Methionine	C_5H_9NOS	131.040485	131	0.040485	0.309
E	Glutamic acid	$C_5H_7NO_3$	129.042593	129	0.042593	0.330
S	Serine	$C_3H_5NO_2$	87.032028	87	0.032028	0.368
N	Asparagine	$C_4H_6N_2O_2$	114.042927	114	0.042927	0.377
G	Glycine	C_2H_3NO	57.021464	57	0.021464	0.377
Y	Tyrosine	$C_9H_9NO_2$	163.063320	163	0.063320	0.388
W	Tryptophan	$C_{11}H_{10}N_2O$	186.079313	186	0.079313	0.426
H	Histidine	$C_6H_7N_3O$	137.058912	137	0.058912	0.430
Q	Glutamine	$C_5H_8N_2O_2$	128.058578	128	0.058578	0.458
F	Phenylalanine	C_9H_9NO	147.068414	147	0.068414	0.465
T	Threonine	$C_4H_7NO_2$	101.047679	101	0.047679	0.472
A	Alanine	C_3H_5NO	71.037114	71	0.037114	0.523
P	Proline	C_5H_7NO	97.052764	97	0.052764	0.544
R	Arginine	$C_6H_{12}N_4O$	156.101111	156	0.101111	0.648
V	Valine	C_5H_9NO	99.068414	99	0.068414	0.691
K	Lysine	$C_6H_{12}N_2O$	128.094963	128	0.094963	0.742
I	Isoleucine	$C_6H_{11}NO$	113.084064	113	0.084064	0.744
L	Leucine	$C_6H_{11}NO$	113.084064	113	0.084064	0.744

Figure 7. A portion of the HCD MS^2 spectrum of the 18+ charge state of SILu Lite mAb Fd Subunit near the precursor *m/z*. This region of the exhibits two series of “picket fence” product ion *m/z* peaks. One series has 1 Th spacing (red dots) and second series has 0.5 spacing (blue dots).



CONCLUSIONS

- “Picket Fence” *m/z* peak series in MS^2 spectra are produced by the constructive interference of the superposition of multiple isotopic *m/z* peak envelopes of relatively highly charged product ions. They are observed in the regions of high product ion m/z peak density in the vicinity the *m/z* values of the intact precursor and any intact charge reduced product ions.
- Amino acid composition and distribution in a polypeptide’s sequence and preservation of relatively large product ion masses after completion of the dissociation step determines if a particular polypeptide’s MS^2 spectrum will likely exhibit “picket fence” *m/z* peak series.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING

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