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Using dissociation energies to predict observability of b- and y-peaks in mass spectra of short peptides. II. Results for hexapeptides with non-polar side chains

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Abstract

RATIONALE—The hypothesis that dissociation energies can serve as a predictor of observability of b- and y-peaks is tested for seven hexapeptides. If the hypothesis holds true for large classes of peptides, one would be able to improve the scoring accuracy of peptide identification tools by excluding theoretical peaks that cannot be observed in practical product ion spectra due to various physical, chemical or thermodynamic considerations.

METHODS—Product ion m/z spectra of hexapeptides AAAAAA, AAFAAA, AAFAAA, AAFAAA, AAFAAA, AAFAAA, AAFAAA and AAFAAA have been acquired on a Finnigan LTQ XL mass spectrometer in the collision-induced dissociation (CID) activation mode on a grid of activation times 0.05 to 100 ms and normalized collision energy 10 to 35%. Dissociation energies were calculated for all fragmentation channels leading to b- and y-fragments at the TPSS/6–31G(d,p) level of the density functional theory.

RESULTS—It was demonstrated that the m/z peaks observed in the product ion spectra correspond to the fragmentation channels with dissociation energies below a certain threshold value. However, there is no direct correlation between the most intense m/z peaks and the lowest dissociation energies. Using the dissociation energies, it was predicted that out of 63 theoretically possible peaks in the b- and y-series of the seven hexapeptides, 19 should not be observable in practical spectra. In the experiments, 24 peaks were not observed, including all 19 predicted.

CONCLUSIONS—Dissociation energies alone are not sufficient for predicting ion intensity relationships in product ion m/z spectra. Nevertheless, the present data suggest that dissociation energies appear to be good predictors of observability of b- and y-peaks and potentially very useful for filtering theoretical peaks of each candidate peptide in peptide identification tools.

One of the potential applications of molecular-level studies of short peptide fragmentation mechanisms would be *in silico* predictions of relative intensities of various m/z peaks in a product ion mass spectrum of a given peptide. Ion intensity relationships, if they can be

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SUPPORTING INFORMATION

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correctly predicted, would definitely improve selectivity and reliability of peptide identification tools.

At the present level of computing power, a comprehensive computational analysis of all possible fragmentation pathways, even for diglycine, is not an easy task.^[1] Moreover, accurate predictions of ion intensity relationships would also require detailed knowledge of experimental conditions (which is not always available) and also a satisfactory theoretical description of other physical processes (collisional heating, electronic excitations, thermalization, etc.).^[2–7]

Although accurate prediction of relative ion intensities remains a task for the future, we believe that one already can take advantage of the achieved understanding^[8–21] of peptide fragmentation mechanisms. A practical way to do so would be to introduce a filtering of the theoretical peaks of candidate peptides before scoring their similarity with the given experimental spectrum.

In most of the peptide identification tools, all theoretically possible b- and y-peaks of a candidate peptide are scored (often with equal weights) against the peaks present in the experimental mass spectrum. The similarity scores can be made much more accurate if the scoring function does not take into account theoretical peaks which cannot be observed in real-life product ion spectra due to various physical, chemical, or thermodynamic reasons. In order to implement such a filtering one needs to choose a physical quantity which would be experiment-independent and relatively easily computable, but which still could serve as a reliable predictor of observability of b- and y-peaks.

In our previous paper^[22] we suggested that the role of this observability predictor can be played by dissociation energy. We demonstrated, using seven tetrapeptides as examples, that the m/z peaks observed in experimental mass spectra correspond to the fragmentation channels with the lowest dissociation energies, and one can choose a certain cut-off energy separating observable peaks from unobservable ones. In particular, choosing 50 kcal/mol as the cut-off for dissociation energy, allowed us to correctly predict observability of 40 out of 42 theoretically possible b- and y-peaks in product ion spectra of those seven tetrapeptides.

In this follow-up work we present results on employing dissociation energies for predicting observability of b- and y-peaks in product ion spectra of seven hexapeptides, AAAAAA, AAFAAA, AAFAAA, AAFAAA, AAFAAA, AAFAAA and AAFAAA. We calculated dissociation energies for 63 theoretically possible fragmentation channels which result in either b- or y-fragments. We also acquired product ion spectra of these seven hexapeptides in a wide range of collision energies and activation times. Using the same cut-off value for the dissociation energies as we did for tetrapeptides (50 kcal/mol), we predicted that in total 44 b- and y-peaks can be observed in the experimental spectra and 19 peaks should not be observed. In experiments, we observed 39 peaks, all of which were predicted as observable. More importantly for the filtering purposes, none of the 19 peaks classified as unobservable had in experimental spectra intensity greater than 1%. Therefore, our new data support the hypothesis that dissociation energies can serve as a reliable predictor of observability of b- and y-peaks in product ion m/z spectra of short peptides with non-polar side chains.

EXPERIMENTAL

The experimental set-up was identical to the one used in our experiments with tetrapeptides.^[22] Namely, experiments were performed on a Finnigan LTQ XL mass spectrometer in the collision-induced dissociation (CID) activation mode. The product ion spectra were acquired on a grid of activation times t_a varying from 0.05 to 100 ms and

normalized collision energy (NCE) varying from 10 to 35%. Solutions (10 pmol/ μ L) of AAAAAA, AAFAAA, AAAVAA, AAFAAA, AAVAAA, AAFFAA and AAVVAA (Princeton Biomolecules Corp., Langhorne, PA, USA, HPLC purified to 97% based on certificates of analysis) were infused into an ESI source using a syringe pump at a flow rate of 3 μ L/min. The spray voltage and ion transfer tube temperature were set at 4.0 kV and 275°C, respectively. The automatic gain control (AGC) was turned on to control the total numbers of ion population in the ion trap. The effective resolution in product ion spectra corresponded to peak widths at half height of 0.7 m/z units; the isolation width for precursor ions was 2 m/z units; the isolation time was automatically controlled by the AGC target of 1E4 (all manufacturer's defaults). The mass spectra were acquired in centroid mode. For our infusion experiments using commercially purified peptides at high concentrations (10 pmol/ μ L), reliable acquisitions in centroid mode and high signal-to-noise ratios ($\sim 10^4$ to 10^5) were achieved based on high ion statistics as well as averaging over tens to hundreds of consecutive scans. In addition, for AAAAAA we acquired spectra on a finer grid of activation times in the interval from 0.03 to 4 ms at 12.5 and 25% of NCE.

COMPUTATIONAL

As in our previous study,^[22] numerical calculations were carried out at the TPSS/6–31G(d,p) level of the density functional theory (DFT) with the use of Gaussian 09 software package^[23] on the Biowulf computer cluster at the National Institutes of Health.

The configuration space of the peptides and their fragments was searched extensively and the geometries with the lowest energies were identified for each protonation site of the precursor ions and for all charged and neutral fragments. Normal vibration modes were calculated for each geometry to determine the zero point energy correction.

Dissociation energy is defined as a difference between the energy of a physical system in the final state and its energy in the initial state. In the case of fragmentation of singly charged peptides, the energy of the final state is the sum of the energies of the charged and neutral fragments, while the energy of the initial state is simply the energy of the precursor peptide E_{MH^+}

$$E_{b_n} \equiv (E(b_n^+) + E(y_{\kappa-n}^0)) - E_{MH^+} \quad (1)$$

$$E_{y_n} \equiv (E(b_{\kappa-n}^0) + E(y_n^+)) - E_{MH^+} \quad (2)$$

where E_{b_n} and E_{y_n} are dissociation energies for b_n^+ - and y_n^+ -peaks, respectively; κ is the number of residues in the parent peptide ion; $E(b_n^+)$ and $E(y_n^+)$ are the energies of the charged b_n^+ - and y_n^+ -fragments, and $E(y_{\kappa-n}^0)$ and $E(b_{\kappa-n}^0)$ are the energies of their neutral counterparts.

The ratio of intensities I_1 and I_2 for any pair of fragmentation channels 1 and 2 with dissociation energies E_1 and E_2 is assumed to be predicted from calculated energy differences (see the first part of our study^[22] for a discussion of the assumptions and for references):

$$\ln \left(\frac{I_1}{I_2} \right) \approx \left(\frac{E_2 - E_1}{kT_{\text{eff}}} \right), \quad (3)$$

where k is the Boltzmann constant and T_{eff} is the effective temperature. Let us note that it is very hard to define T_{eff} rigorously under collisional activation conditions. However, this

parameter is in fact not relevant to the application of the suggested approach where the key adjustable parameter is the cut-off dissociation energy for observable product ions.

There are κ possible fragmentation channels resulting in formation of b-fragments (if water loss channel is included) and $\kappa - 1$ channels resulting in formation of y-fragments. For a hexapeptide this amounts to 11 theoretically possible b- and y-fragments. For seven hexapeptides, there are, correspondingly, 77 theoretically possible fragments. However, the b_1^+ -fragments are not stable, so these fragmentation channels can automatically be classified as unobservable. Further, m/z of the y_1^+ -fragments were outside the detection range of the LTQ XL spectrometer used in our experiments. Thus, there are 63 fragmentation channels for which dissociation energies should be calculated in order to predict observability of the corresponding b- and y-peaks in the experimental product ion spectra.

Finally, we note that knowledge of dissociation pathways is very important. As is discussed in the first part of our study,^[22] the geometries of the charged and neutral fragments to be used for calculating the dissociation energies are not always the lowest energy geometries. For example, neutral b-fragments can be oxazolone or diketopiperazine derivatives. The latter structures have much lower energies and therefore much smaller dissociation energies. However, according to the b_x-y_z fragmentation pathway,^[9] oxazolone structures are actually formed immediately after the amide bond cleavage, due to a very high transition barrier involved in the diketopiperazine fragmentation pathway.^[9,24,25] Thus, oxazolone structures were used when calculating energies of b-fragments.

The energies and geometries of the precursor ions and the charged and neutral fragments which were used to determine the dissociation energies for all seven hexapeptides are provided in the Supporting Information.

RESULTS AND DISCUSSION

Dissociation energies for all fragmentation channels leading to formation of b- and y-ions are presented in Table 1. For all seven hexapeptides, the water loss fragmentation channel $MH^+ \rightarrow b_6^+ + H_2O$ has the lowest dissociation energy (as for all seven tetrapeptides considered in the previous paper^[22]), followed by the $MH^+ \rightarrow y_4^+ + b_2^0$, $MH^+ \rightarrow b_5^+ + y_1^0$ and $MH^+ \rightarrow b_4^+ + y_2^0$ processes.

Experimental intensities for b–y-series peaks at $t_a = 10$ ms and NCE = 12.5% are summarized in Table 2. The presented values are typical for collision conditions in which most of the precursor ions are fragmented. When the collision energy and/or activation time are increased further, the intensities of various m/z peaks remain largely unchanged, as illustrated in Table 3 for the example of hexaalanine. Although intensities of the dominant peaks, at their maximum values, agree reasonably well with the intensities previously reported by Harrison and Young,^[10] we do not observe sequential fragmentation to the same extent as these authors.

A comparison of Tables 1 and 2 clearly demonstrates that there is no direct correspondence between the order of dissociation energies and the order of the corresponding ion intensities in experimental spectra. The most striking disagreement is between small dissociation energies of the $MH^+ \rightarrow y_4^+ + b_2^0$ fragmentation channels and quite low intensities of y_4^+ peaks for all seven hexapeptides. The intensities of b_6^+ peaks are also lower than one would expect based on low dissociation energies of the water loss fragmentation channel. For

tetrapeptides, we similarly observed lower than expected intensities of water loss b_4^+ peaks.^[22]

We attribute this discrepancy mainly to the importance of entropy contributions which are completely neglected in Eqn. (3). There also might be a number of other factors influencing the observed intensities. Consecutive fragmentation leads to an increase in relative abundances of smaller b-ions (for instance, appearance of the observed b_2^+ ions can be attributed to this effect). Kinetics can also be of importance, opening up, in particular, non-thermally driven fragmentation pathways. Non-thermal fragmentation can explain, for instance, the low intensity of y_4^+ peaks, because, in this case, precursor ions, in accordance with the b_x-y_z fragmentation pathway,^[9] must be protonated at N₃ and such configurations seem to have comparatively high energies, as demonstrated by Irikura and coworkers for hexaalanines.^[17] (The energies of intermediate states in a dissociation process are irrelevant if the system is thermalized.)

However, our data suggest that all these various factors are secondary, at least for the gentle CID used in our experiments. The data from Table 3 demonstrate that dissociation energies correlate well with the order of appearance of different m/z peaks in mass spectra as the activation time is increased (again, with the exception of the suppressed $MH^+ \rightarrow y_4^+ + b_2^0$ channel). Indeed, the b_6^+ and a less intense b_5^+ peaks are present in product ion spectra even at the shortest activation times, probably due to fragmentation occurring at the ion isolation and mass analysis stages of the ion trap analytical cycle. At $t_a = 0.9$ ms, the b_4^+ peak appears, followed by the y_4^+ peak at $t_a = 1.1$ ms. Finally, b_3^+ and y_3^+ peaks appear at $t_a = 1.3$ ms.

The data from Tables 1 and 2 show that dissociation energies also correlate well with the observability of the corresponding b- and y-peaks. For the seven considered hexapeptides, dissociation energies for 19 fragmentation channels are greater than 50 kcal/mol. None of the corresponding 19 b- and y-peaks had intensities greater than 1% in typical experimental spectra. On the other hand, the dissociation energies corresponding to all 39 observed b- and y-peaks are lower than 50 kcal/mol. Thus, dissociation energies allow one to correctly predict observability of 58 out of 63 fragmentation channels.

Similarly, for tetrapeptides,^[22] dissociation energies of 14 fragmentation channels are greater than 50 kcal/mol and none of the corresponding m/z peaks was observed. Dissociation energies corresponding to all 26 observed peaks were lower than 50 kcal/mol. Thus, dissociation energies allowed us to correctly predict observability of 40 out of 42 fragmentation channels.

Two more circumstances are worth mentioning. First, the same energy cut-off of 50 kcal/mol works very well for both hexa- and tetrapeptides. Second, predictions based on dissociation energies are conservative, i.e. fewer m/z peaks are predicted to be unobservable than in fact are.

It appears, therefore, that dissociation energies can potentially be very useful for filtering peaks in the theoretical spectra of candidate peptides in peptide identification tools. Dissociation energies can be used to divide theoretically possible b- and y-peaks into 'observable' and 'unobservable' ones. The accuracy and reliability of peptide identification can be improved if only 'observable' theoretical m/z peaks of a candidate peptide are scored against a given experimental product ion spectrum.

As an example, we applied the scoring algorithm used in the RAId peptide identification tool^[26] to obtain similarity between the theoretical mass spectrum of hexaalanine (all b- and

y-peaks are assumed to be equally observable) and the real hexaalanine mass spectrum acquired at $t_a = 10$ ms and NCE = 12.5%. The calculated RAId score was 1.765. After removing b_1^+ , b_2^+ , y_1^+ , y_2^+ , and y_5^+ peaks (classified as 'unobservable' based on the corresponding dissociation energies) from the theoretical mass spectrum, the RAId score increased to 3.16. We note that, even though such re-scoring would potentially lead to much more significant E-values for correct peptides, its global implementation is still far from practice because of the substantial computational efforts needed to obtain dissociation energies.

To conclude, our current and previous^[22] data suggest that dissociation energies are likely to be good predictors of observability of b- and y-peaks in mass spectra of short non-polar peptides. It would be of great interest to check how well dissociation energies can predict observability of b- and y-peaks for other types of peptides, particularly with charged side chains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Dissociation energies (kcal/mol) for fragmentation channels resulting in formation of b- and y-series fragments. TPSS/6–31G(d,p) model chemistry is used for geometry optimization and energy calculations. Zero point energy corrections are taken into account

Peptide	Fragments									
	b_2^+	b_3^+	b_4^+	b_5^+	b_6^+	y_1^+	y_2^+	y_3^+	y_4^+	y_5^+
AAAAAA	53.2	49.7	44.7	39.3	35.2	63.1	52.8	47.8	37.8	51.0
AAAVAA	52.8	48.9	42.1	38.2	34.5	62.7	51.3	46.6	36.6	51.2
AAVAAA	52.3	46.4	42.4	37.6	33.5	60.5	52.2	45.7	36.3	49.1
AAVVAA	52.7	46.4	40.6	37.5	33.3	60.7	51.7	45.3	36.4	49.7
AAAFAA	54.9	49.5	44.6	40.0	34.7	64.8	54.1	47.3	37.2	51.7
AAFAAA	52.5	47.7	42.2	37.4	34.1	61.6	52.9	47.6	34.9	51.1
AAFFAA	54.4	48.0	42.5	38.1	33.8	63.3	54.6	47.5	34.8	50.6

Table 2

Intensities of various m/z peaks (per cent of the total intensity). Activation time is 10 ms. Normalized collision energy is 12.5%. a_n^+ denotes $a_n^+ - 17$ fragments. In the column 'other' the sum of intensities of all other m/z peaks is given

Peptide	Fragments													
	b_2^+	b_3^+	b_4^+	b_5^+	b_6^+	y_2^+	y_3^+	y_4^+	y_5^+	a_4^+	a_4^*	a_5^+	a_5^*	other
AAAAAA	0.5	2.1	12.6	59.0	6.3	0.2	1.0	1.4	0.1	2.9	0.9	6.5	2.7	3.0
AAAVAA	0.6	3.4	23.3	42.4	5.3	0.1	1.6	0.7	0.0	10.1	0.9	6.4	1.4	3.7
AAVAAAA	0.7	2.9	9.8	56.5	6.3	0.2	1.9	1.9	0.1	4.9	0.6	8.7	2.0	3.4
AAVVAA	0.3	3.5	16.6	50.9	5.9	0.1	3.5	0.7	0.1	7.1	0.4	5.8	1.1	3.6
AAAFAA	0.1	1.2	13.1	50.6	5.5	0.1	3.0	1.6	0.1	5.8	1.6	7.3	2.7	6.7
AAFAAA	0.1	2.5	11.4	60.3	6.6	0.1	0.5	2.3	0.1	2.1	0.6	6.8	2.3	4.1
AAFFAA	0.0	1.9	11.1	56.0	5.9	0.0	2.0	2.5	0.1	4.4	0.8	6.9	2.2	6.0

Table 3

Intensities of various m/z peaks (per cent of the total intensity) in product ion spectra of AAAAAA. Normalized collision energy is 12.5%. a_n^* denotes $a_n^+ - 17$ fragments

t_a (ms)	Fragments												
	b_2^+	b_3^+	b_4^+	b_5^+	b_6^+	y_2^+	y_3^+	y_4^+	y_5^+	a_4^+	a_5^+	a_5^*	MH ⁺
0.1				0.09	0.22								99.67
0.5				0.09	0.23								99.66
0.9			0.01	0.14	0.23								99.61
1.1			0.06	0.46	0.28						0.01		99.15
1.3		0.02	0.19	1.24	0.36		0.01				0.03		98.06
1.5	0.01	0.04	0.44	2.59	0.53		0.04	0.01		0.01	0.07	0.02	96.12
2.0	0.02	0.10	1.01	5.62	0.75		0.08	0.12		0.11	0.22	0.07	91.48
3.0	0.11	0.54	4.58	23.84	2.74	0.04	0.38	0.53	0.02	0.72	1.41	0.47	63.80
4.0	0.27	1.18	8.47	41.34	4.51	0.10	0.69	0.99	0.05	1.60	3.40	1.34	34.06
5.0	0.37	1.71	10.34	49.37	5.33	0.15	0.86	1.17	0.06	2.28	5.00	2.08	18.30
6.0	0.43	1.99	11.55	53.29	5.71	0.17	0.94	1.30	0.07	2.70	5.98	2.56	9.61
8.0	0.49	2.19	12.39	57.14	6.10	0.20	0.98	1.38	0.07	2.96	6.72	2.93	2.37
10	0.47	2.14	12.60	59.02	6.33	0.20	1.00	1.42	0.08	2.93	6.47	2.67	0.76
14	0.42	1.90	12.44	62.41	6.70	0.17	1.02	1.49	0.07	2.67	5.44	1.99	0.14
20	0.35	1.59	12.42	65.62	7.03	0.12	1.05	1.52	0.06	2.29	4.26	1.30	0.02
30	0.29	1.29	12.48	68.19	7.43	0.10	1.09	1.56	0.05	1.88	3.16	0.76	
50	0.24	1.07	12.49	70.52	7.78	0.06	1.06	1.55	0.05	1.42	2.17	0.42	
90	0.18	0.87	12.34	72.51	8.06	0.04	1.03	1.58	0.05	1.03	1.37	0.21	