A-, B-, C-, D-, W-, Y- and Z-Ions of Peptides and Proteins Generated Simultaneously by MALDI In-Source Decay

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Overview

Purpose: Evaluation of 1,5-diamino naphthalene (1,5-DAN) matrix for sequencing of peptides and proteins analyzed on hybrid ion trap-Orbitrap mass spectrometer equipped with a MALDI ion source.

Methods: 1,5-diamino naphthalene (1,5-DAN), ACTH (18-39) was prepared as a saturated solution in 50% ACN/50% water/0.1%TFA, mixed in a 1:1 ratio with ACTH, insulin-B chain (oxidized) and chicken lsozmye, spotted on a stainless steel target and air dried at ambient temperature. Full-scan MS spectra were acquired on a hybrid ion trap-Orbitrap mass spectrometer. A selection of averaged spectra (between 10 and 25) scans are displayed in the figures.

Results: The ability of 1,5-DAN to produce a mixture of the four most common low-energy produced sequence ions (c-, z-, b-, and y-type ions) is demonstrated in four examples: a mid-size peptide at mass 2464 (ACTH fragment 18-36), a larger peptide at mass 3495 (oxidized insulin-B chain) and a 14.3 kDa small-size protein (lysozyme). All fragments are detected in FTMS full scan without use of precursor isolation or excitation energies. For the MALDI in-source decay (ISD) spectra of ACTH and insulin-B chain, the ISD fragment ions are observed next to the intact precursor in the same scan. For insulin-B chain, all series of a-, b-, y-, c- and z-ions, as well as a number of w- and d-ions, are detected simultaneously in a single mass spectrum covering the full 30 amino acid sequence. The analysis of native lysozyme results in fragment ions that contain free Cys-residues, thus demonstrating the reducing nature of the matrix.

Introduction

MALDI-produced fragment ions can be generated using a 1,5-diamino naphthalene (1,5-DAN) matrix. This matrix enables rapid reduction of disulfide bonds (1) and N-terminal and C-terminal sequencing of peptides and small proteins (2) . 1,5-DAN matrix has been shown to produce a significant amount of ISD fragments in axial time-of-flight (TOF) systems (2). MALDI ISD fragment-ion production was first described in the mid 1990's by Brown et al. in studies employing delayed extraction in axial TOF systems (3, 4, 5). A model explaining the underlying ISD mechanism in MALDI was proposed by Takayama ($N\text{-}C_\alpha$ bond cleavage of peptide backbone via hydrogen abstraction), and its parallels to ECD were given (6). Using 1,5-DAN, Demeure et al. observed entire c- and z-type sequence ladders of sequence-specific fragment ions simultaneously using axial TOF geometry (2). In contrast to axial TOF which presents relatively low pressure during sampling at the sample plate, the MALDI ion source used in this study liberates the MALDI-produced fragment ions into a region of significantly higher pressure (75 mTorr) (7), a collisional-cooling regime. Ions, in this case the ISD fragment ions, are detected in a Orbitrap detector.

For the first time, we show 1,5-DAN matrix applied to a collisional-cooling interface and Orbitrap detection. With Orbitrap mass analysis, ISD fragment ions of peptides or proteins and entire sequence tags can be observed. Further, a-, b-, c-, y- and z- type fragment ions are observed in a spectrum simultaneously. In a middle-down or top-down approach, sequences of larger peptides (< 4kD), and N-terminal or C-terminal sequence tags of intact proteins above 50 kD, can be obtained and assigned.

Methods

Reagents: 1,5-diamino naphthalene (1,5-DAN), ACTH (18-39), Insulin B chain (oxidized) and chicken lsozmye (Sigma-Aldrich, Steinheim, Germany).

Matrix and analyte solutions: 1,5-DAN was used as matrix and prepared as a saturated solution in water/actenotrile 50/50 v/v with 0.1% TFA and centrifuged before usage (2). The 1,5-DAN solutions were always prepared shortly before MS experiments because of its instability in acetonitrile. Peptides and proteins were dissolved in HPLC grade water at concentrations of 5 pmol/µL and mixed in a ratio 1:1 with 1,5-DAN. 0.5 µLof each analyte/matrix mixture was spotted on a stainless-steel target and air dried at ambient temperature.

Mass Spectrometry: All experiments were performed on a Thermo Scientific MALDI LTQ Orbitrap XL hybrid mass spectrometer, operating at intermediate pressure (75 mTorr) (7). Spectra were acquired from single scans or as averages of up to 3 microscans. Averages of a few scans (between 10 to 25) are displayed. A single µscan can easily meet a target "number of charges" of 1e6 in the Orbitrap™ detector with 50 laser shots.

Data processing: Raw data were processed with "Xtract" to obtain the monoisotpic mass lists that were submitted to ProSight PTM, https://prosightptm.northwestern.edu/, for fragment ion matching of known sequences.

Results

Figure 1 is the mass spectrum obtained from 1,5-DAN. Besides monomeric radical and protonated species, ions representing dimeric and trimeric structures are detected. The ability of the matrix to produce a mixture of the four most common low-energy produced sequence ions --- c-, z-, b- and y-type ions -- is demonstrated in three examples: a mid-size peptide at mass 2464 (Figure 2, ACTH fragment), a larger peptide at mass 3495 (Figure 3, oxidized bovine insulin-B chain) and a 14.3 kDa protein (Figure 4, chicken lysozyme). All fragments are detected in the MALDI ISD full-scan MS spectrum next to the intact precursor (Figures 2 and 3).

Fragmentation is rich and spontaneous without any further energies and fragmentation reactions required. In a single scan, a series of a-, b-, c- and z-ions are detected simultaneously. The reducing nature of the matrix is highlighted in the analysis of native lysozyme, which contains three disulfide bonds. The disulfide bonds are apparently reduced by the matrix, making the detection of cysteine containing fragments possible.

FIGURE 1. MALDI ISD full-scan MS spectrum of 1,5-DAN (M = 158.08385), and proposed peak and structure assignments of the major species detected: the radical cation monomer, dimer and trimer in a mixed population with their protonated species and isotope patterns.

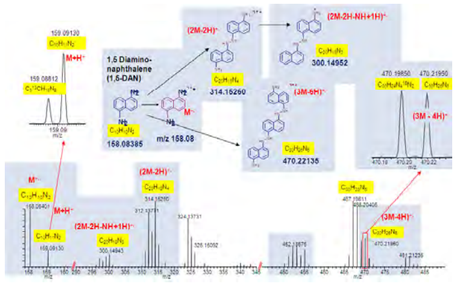


FIGURE 2. MALDI ISD full-scan MS spectrum of ACTH (18-39) (M = 2464). The dominant series of b- and c-type ions are detected and complemented by a few a- and y-type ions.

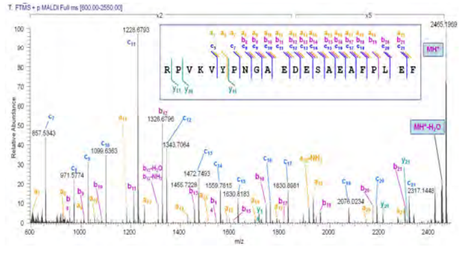
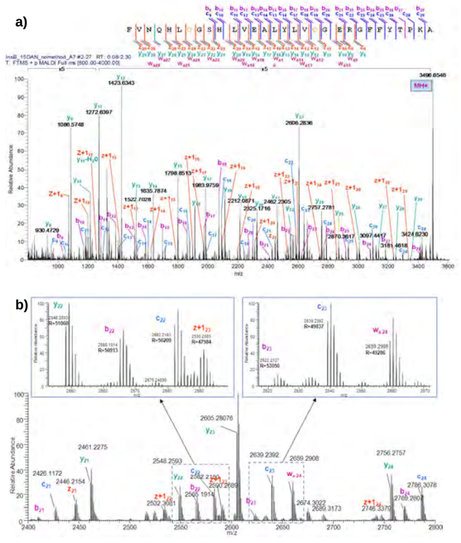


FIGURE 3. MALDI ISD full-scan MS spectrum of insulin-B chain (oxidized) (M = 3495). All four series of b-, c-, y- and z-type ions are nearly completely detected, thus covering the full peptide sequence.
a) Overview of the FTMS full-scan MS spectrum, average of 26 single scans acquired with resolving power 100,000 @ m/z 400.
b) Inset highlights mass resolution of approximately RP=50,000 for m/z 2550 (left) and m/z 2650 (right). Monoisotopic masses are assigned (MH^+) and fragment ion types are given.
c) Fragment ion types and fragment ions observed for the insulin-B chain using MALDI ISD with 1,5-DAN matrix.

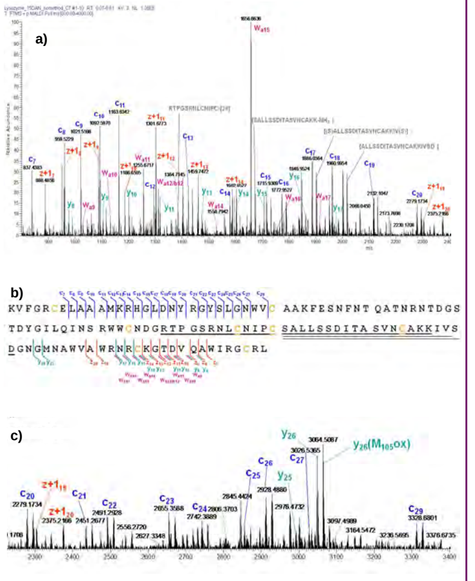


c)

w	d	a	b	c			y	z	z+1
					1	C	29	...	
					2	V	28	3347.58918	3332.57547
					3	N	27	3248.51581	3233.50510
					4	Q	26	3134.47609	3117.44808
					5	H	25	3006.41708	2991.40396
					6	L	24	2869.35870	2854.34706
					7	C*	23	2756.27570	2741.29051
					8	G	22	2605.28072	2590.26891
					9	S	21	2548.25929	2533.24810
					10	H	20	2461.22751	2446.21538
					11	L	19	2324.16831	2309.15675
					12	V	18	2211.08410	2196.07231
					13	E	17	2112.01578	2097.00450
					14	A	16	1982.97272	1967.96140
					15	L	15	1911.93541	1896.92469
					16	Y	14	1798.85126	1783.84002
					17	L	13	1635.78740	1620.77645
					18	V	12	1522.70279	1507.69200
					19	C*	11	1423.63426	1408.62339
					20	G	10	1272.63960	1257.62901
					21	E	9	1215.61792	1200.60669
					22	R	8	1086.57484	1071.56375
					23	G	7	930.47294	914.45289
					24	F	6	873.45117	857.43102
					25	F	5		
					26	Y	4		
					27	T	3		
					28	P	2		
					29	K	1		
					30	A	0		

w	d	a	b	c
			890.38312	907.40834
		919.40965	947.40511	964.43142
		1006.44207	1034.43741	1051.46382
		1143.50179	1171.49677	1188.52335
		1256.58636	1284.58104	1301.60775
		1355.65461	1383.65023	1400.67664
		1484.69905	1512.69323	1529.71961
		1555.73594	1583.73053	1600.75706
		1668.81978	1696.81519	1713.84131
		1831.88398	1859.87887	1876.90521
		1944.96829	1972.96315	1989.98957
		2044.03620	2072.03082	2089.05833
		2195.05651	2223.02520	2240.05124
			2280.04803	2297.07416
		2381.09542	2409.08892	2426.11723
		2537.19736	2565.19139	2582.21803
			2622.21073	2639.23916
		2741.29051	2769.28068	2786.30770
		2888.35488	2916.34847	2933.37588
		3051.41820	3079.41141	3096.43799
		3152.47274	3180.45887	...
				3294.53831
			3405.60516	3422.63646
			...	...

FIGURE 4. MALDI ISD full-scan MS spectrum of intact lysozyme (M=14.3 kDa) and fragment ion annotation of c-, z-, b- and y-ions. A large portion of the protein near the N-terminus is covered by a complete c-ion series. The C-terminus is covered by a smaller series of y- and z-ions. The middle section of the sequence is represented by three internal fragments. The c- and y- ion series demonstrates the reducing nature of 1,5-DAN because native lysozyme was spotted. Nevertheless, the reduced cysteines are represented in all fragments except for y/z7-y/z14.
a) FTMS full-scan spectrum between *m/z* 800 – 2300 (spectrum continued in c).
b) Sequence of lysozyme with observed fragment ions and types.
c) FTMS full-scan spectrum between *m/z* 2100 - 3400, (continued from a).



Another characteristic of 1,5-DAN, observed in the analysis of lysozyme in its native form, is its reducing nature. Lysozyme contains four disulfide bonds which are apparently reduced by the matrix (6-127, 30-115, 64-80, 76-94 in the protein sequence without a signal peptide shown in Figure 4b). This allows detection of cysteine-containing fragment ions. Fukuyama et al. (1) performed experiments which suggested that the reduction reaction occurs *in vacuo* and not during sample preparation in the liquid phase. Analysis of the spectrum obtained from native lysozyme confirms the presence of six out of the eight possible free cysteine residues in the sequence. Fragment ions containing Cys30 and Cys64 were not observed. These two cysteine residues are not connected via a disulfide bond in the protein's native form. Because all other cysteine residues are contained with the fragment ions detected, it can be concluded that all four disulfide bonds were reduced by 1,5-DAN. All observed c-ions contain cysteine at position six (Cys6), and all z- and b- ions contain Cys127 and some Cys115. All internal sequence ions contain Cys76, Cys80 or Cys94. Intact disulfide bonds would either prevent fragmentation or result in cross-linked fragment ions which are very challenging to assign.

Conclusion

- We have demonstrated the feasibility of top-down sequencing of large peptides and proteins using 1,5-DAN matrix in combination with ISD fragmentation in a MALDI interface with collisional cooling, and high-resolution accurate-mass detection in an Orbitrap mass spectrometer.
- Full MS spectra of peptides and proteins acquired with 1,5-DAN on the MALDI LTQTM Orbitrap XL mass spectrometer are very rich in sequence-specific fragment ions.
- Fragment ions are detected in FTMS full scan mode without precursor selection, resonance excitation or collision induced dissociation.
- Series of c- and z-type ions, as well as b-, and y-type ions complemented by a-, d- and w-type ions and internal fragment ions, are obtained simultaneously when collisonal cooling during MALDI ion production is used combination with accurate mass measurement of sequence-specfic fragment ions in the Orbitrap mass analyzer.
- ISD fragmentation occurs intrinsically as part of the MALDI process and is particularly pronounced using the 1,5-DAN matrix. It is not pronounced in matrices such as 2,5 DHB or CHCA.
- A wealth of information about a peptide and a protein can be obtained in a very short time when using this top-down approach. As such, it offers an appealing alternative to top-down sequencing by ESI.
- Disulfide bonds are reduced by the 1,5 DAN matrix during ionization, enabling detection of peptides containing unmodified cysteine residues. Moreover, unmodified cystein residues – as in the case of lysozyme – promote the formation and detection of very abundant w-type fragment ions (personal communication by Guillaume van der Rest, Ecole Polytechnique, Paris, France).
- The MALDI ISD approach enables sequencing of entire peptides, as well as N- and C-terminal sequencing of proteins.

References

1. Y. Fukuyama, S Iwamoto, K. Tanaka, Rapid sequencing and disulfide mapping of peptides containing disulfide bonds by using 1,5-DAN as a reductive matrix, *J Mass Spectrom.* 41: 191 – 201 (**2006**).
2. K. Demeure, L. Quinton, V. Gabelica, E. De Pauw, Rational selection of the optimum MALDI matrix for top-down proteomics by in-source decay. *Anal Chem.* 79(22):8678-85 (**2007**).
3. R.S. Brown and J.J. Lennon, Mass Resolution Improvement by Incorporation of Pulsed Ion Extraction in a Linear Time-of- Flight Mass Spectrometer, *Anal. Chem.*, 67(13), 1998 (**1995**).
4. R.S. Brown and J.J. Lennon, Sequence Specific Fragmentation of Matrix Assisted Laser Desorbed Protein/Peptide Ions, *Anal. Chem.*, 67(21), 3990 (**1995**).
5. R.S. Brown, B.L. Carr and J.J. Lennon, Factors Influencing the Observed Fast Fragmentation of Peptides in Matrix Assisted Laser Desorption, *JASMS*, 7, 225 (**1996**).
6. M. Takayama, $N\text{-}C_\alpha$ bond cleavage of peptide backbone via hydrogen abstraction, *JASMS* 12, 1044 -1049 (**2001**).
7. K. Strupat, V. Kovtoun, H. Bui, R. Viner, G. Stafford, S. Horning, MALDI Produced Ions Inspected with a Linear Ion Trap-Orbitrap Hybrid Mass Analyzer *JASMS* 20, 1451-1463 (**2009**).

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