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Free Radical Initiated Peptide Sequencing Using MALDI-TOF/TOF Mass Spectrometry

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ABSTRACT : In this study, matrix-assisted laser desorption/ionization (MALDI) was applied to the TEMPO-assisted FRIPS for the first time. We found that 3-HPA is the optimal matrix for the analysis of *p*-TEMPO-Bz-Sc-peptides, which gives minimal precursor fragmentations. MALDI-TOF/TOF experiments on *p*-TEMPO-Bz-Sc-peptides yielded mainly $[\boldsymbol{a}_n+H]^+$, $[\boldsymbol{z}_n+H]^+$, and $[\boldsymbol{y}_n]^+$-type products, indicating that radical-driven peptide fragmentation occurs in MALDI-TOF/TOF-MS.

Keywords : FRIPS, MALDI-TOF/TOF, mass spectrometry, peptides, radical ion, radical-driven fragmentation

Introduction

Soft ionization methods, such as electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) have enabled the introduction of intact biomolecules into a mass spectrometer.[1-4] As a result, soft ionization-based mass spectrometry methods have become widely used for the identification of proteins and peptides. MALDI time-of-flight mass spectrometry (MALDI-TOF MS) is a very efficient tool that has certain advantages over ESI-based mass spectrometry.[5,6] First, MALDI predominantly generates singly charged ions, allowing easy and direct searches for peptides, *i.e.*, peptide mass fingerprinting (PMF). Second, MALDI does not require rigorous sample separation processes when analyzing complex digested peptide mixtures. These advantages have even rendered MALDI-TOF MS as an effective, emerging tool for microbial identification.[7,8]

Although PMF protein identification is possible with a combination of MALDI-TOF MS and database searches, its use is quite limited to the proteins whose information is only available in the database, and unambiguous identification is often hindered due to a lack of sequence information. Thus, tandem mass spectrometry is often essential for unambiguous protein identification, in particular, for *de novo* peptide and protein sequencing. Therefore, MALDI-TOF/TOF has been widely used for peptide sequencing and protein identification.[9,10]

Recently, the development of radical-based peptide tandem mass spectrometry has attracted significant attention.[11,12] Among the numerous radical-based methods, our group has been interested in the development of the free radical initiating peptide sequencing mass spectrometry (FRIPS MS) method, wherein a radical initiator is introduced into the peptide through chemical conjugation into the peptide's N-terminus.[13-23] A radical site is introduced via collisional activation of the peptide with a radical precursor. Secondary activation of generated peptide radical ions was shown to induce extensive peptide fragmentation. FRIPS MS has potential as a useful tool to investigate protein post-translational modifications as well as disulfide-bond mapping, such as electron capture/transfer dissociation (ECD/ETD).[14,24-26] FRIPS MS method has the characteristic feature of generating ***a***-, ***x***-, ***c***-, and ***z***-type ions that are dominant fragments, as well as minor ***b***- and ***y***-type ions. This fragmentation pattern is quite different when compared with typical collisional activation dissociation.

Currently, our FRIPS MS method has only been used on platforms of ESI-based mass spectrometry but has not been

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studied using an MALDI ion source. In general, hot matrices, such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB), are known as efficient matrices for peptide and protein ionization. However, as we discuss below, the analysis of conjugated peptides with the FRIPS MS approach was not possible with these matrices, thus necessitating the exploration of other matrices.

In this study, MALDI ionization of the conjugated peptides was evaluated using six different matrices, including CHCA, DHB, *super* DHB (mixture of 2,5-DHB and 2-hydroxy-5-methoxybenzoic acid), 2,4,6-trihydroxyacetophenone (THAP), dithranol, and 3-hydroxypicolinic acid (3-HPA). Furthermore, we evaluated whether MALDI-TOF/TOF tandem mass spectrometry is useful for peptide characterization with the FRIPS MS approach.

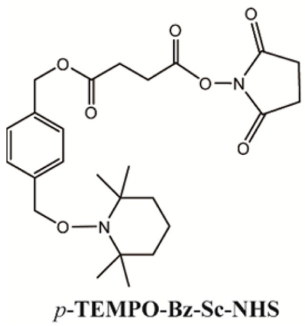
Materials and Methods

Materials

P(ara)-TEMPO-benzyl-succinate-NHS (4-(2,2,6,6-tetramethylpiperidine-1-oxyl) methyl benzyl succinic acid N-hydroxysuccinimide) was used as a radical initiator precursor and was purchased from Futurechem (**Scheme 1**, FC-8502, Seoul, Korea). Note that we used this compound in this study instead of *ortho*-TEMPO-Benzyl-NHS. Detailed information about *p*-TEMPO-Bz-Sc-NHS will be discussed in a forthcoming publication. Peptides ARVYIHP, DRVYIHPFHL (Angiotensin I), and DRVYIHPFHLLVYS were obtained from Sigma (St. Louis, MO, USA), and RVYIHPFHL was custom-synthesized by Anygen (Gwangju, Korea). CHCA, DHB, *super* DHB, THAP, dithranol, and 3-HPA were purchased from Sigma. Only HPLC grade solvents were used in this study. Methanol, water, and acetonitrile (ACN) were purchased from Burdick & Jackson (Ulsan, Korea). Anhydrous dimethyl sulfoxide (DMSO), formic acid (FA), and tetraethylammonium bicarbonate (TEAB) were purchased from Sigma.

Conjugation of peptides with *p*-TEMPO-Bz-Sc-NHS

A peptide solution in DMSO and 2 μL of *p*-TEMPO-Bz-Sc-NHS DMSO (1 mg in 100 μL) were initially mixed in a 1:2 mole ratio, into which 2 μL of TEAB was added. Finally, DMSO was added for a total volume of 30 μL. The resulting reaction mixture was left at room temperature overnight and dried using a SpeedVac. Each MALDI matrix was prepared as a saturated solution in 0.5% FA/ 50% ACN aqueous solution. The loaded MALDI plate contained 1 μL of a 1:1 (v/v) mixture of sample and matrix solution.



Scheme 1. Chemical structure of *p*-TEMPO-Bz-Sc-NHS

Mass spectrometry

Matrix selection and optimization of MALDI-TOF MS experiments were conducted on a Bruker Autoflex Speed series mass spectrometer (Bruker Daltonics, Leipzig, Germany). MALDI-TOF/TOF mass spectra were acquired using either a Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer or an ABI 4800 plus MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA). For the MALDI-TOF MS spectra shown in **Figure 1**, we used the following mass spectrometry parameters: positive mode; ion source 1, +19.05 kV; ion source 2, +16.70 kV; Lens, +8.25 kV; reflector, +21.00 kV; reflector 2, +9.70 kV; collision gas, helium; 5,000 total shots (1,000 laser shots per spot). For the MALDI-TOF MS/MS spectra using a Bruker TOF/TOF instrument, the following

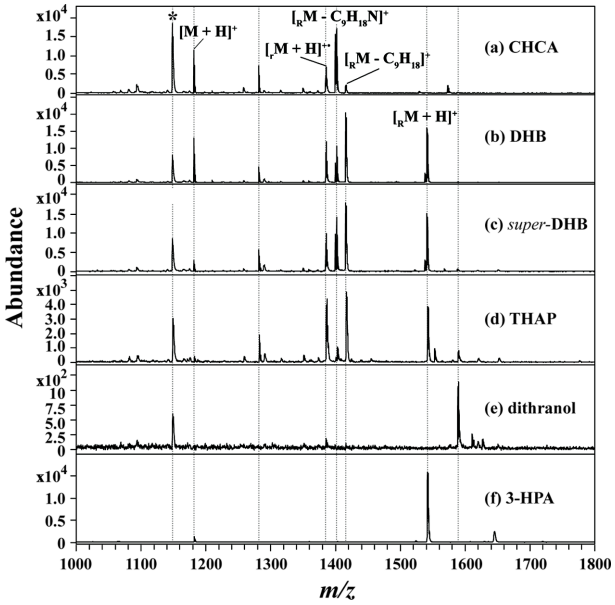


Figure 1. The MALDI-TOF MS spectra obtained using six different matrices to detect *p*-TEMPO-Bz-Sc-RVYIHPFHL at *m/z* 1541: (a) CHCA, (b) DHB, (c) *super* DHB, (d) THAP, (e) dithranol, and (f) 3-HPA. The neutral loss of $C_9H_{18}N$ arose from bond cleavage between the TEMPO oxygen and nitrogen in the *p*-TEMPO-Bz-Sc-RVYIHPFHL. The neutral loss of C_9H_{18} arose from bond cleavage between the TEMPO nitrogen and two quaternary carbons. *: unassigned peak.

parameters were used: positive mode; ion source 1, +7.54 kV; ion source 2, +6.80 kV; lens, +3.52 kV; reflector, +29.50 kV; reflector 2, +14.00 kV; lift 1, +19.00 kV; lift 2, +3.20 kV; collision gas, argon; 10,000 total shots (1,000 shots per spot). For the MALDI-TOF MS/MS spectra using an ABI TOF/TOF mass spectrometer, the following parameters were used: positive-reflectron mode; acceleration voltages, +20 kV; collision cell, +2 kV; collision gas, air; 5,000 total shots (500 shots per spot).

Results and Discussion

Matrix selection

For the optimal MALDI-TOF MS detection of *p*-TEMPO-Bz-Sc-conjugated peptides, a variety of MALDI matrices were evaluated. **Figure 1** shows the resulting MALDI-TOF MS spectra for *p*-TEMPO-Bz-Sc-RVYIHPFHL acquired using the matrices of (a) CHCA, (b) DHB, (c) *super* DHB, (d) THAP, (e) dithranol, and (f) 3-HPA. The ionization efficiency and the degree of the conjugated peptide fragmentation varied significantly depending on the matrix used. CHCA, DHB, and *super* DHB provided excellent ionization efficiency. However, these matrices produced a number of high abundance fragment peaks, even with a low laser power. Specifically, CHCA, DHB, *super* DHB, and THAP matrices yielded two highly abundant peaks of $[_{R}M-C_9H_{18}]^+$ at *m/z* 1414.7 and $[_{R}M-C_9H_{18}N]^+$ at *m/z* 1400.7, where the subscript R represents the *p*-TEMPO-Bz-Sc group attached to the peptide's N-terminus. These two peaks arose from bond cleavage between the TEMPO oxygen and nitrogen, *i.e.*, $[_{R}M-C_9H_{18}]^+$, and between the TEMPO nitrogen and two quaternary carbons, *i.e.*, $[_{R}M-C_9H_{18}N]^+$, in the *p*-TEMPO-Bz-Sc-RVYIHPFHL at *m/z* 1540.9. In particular, CHCA, which is known as the hardest MADLDI matrix, did not show any intact conjugated peptide (see **Figure 1a**).[27-29] We expect that the production of a number of fragments limits the use of MALDI-TOF MS as a tool for protein identification since *m/z* acquisition for the intact peptides within the peptide mixtures are easily hindered due to the presence of other fragments in the mass spectrum. For DHB, *super* DHB, and THAP, we observed both the intact conjugated peptides and fragments (see **Figure 1 b**, **c**, and **d**). In contrast, 3-HPA generated intact conjugated peptide ions as a dominant peak with very minor fragment peaks, although we also observed that this matrix allowed only a few spots for efficient ionization.

The tendency and extent of precursor fragmentation observed in this study could be understood in terms of the "*hard/soft*" or "*hot/cold*" characteristics of the matrices used. Previous studies reported that the MALDI matrix hardness orders are as follows: CHCA >> DHB > sDHB > THAP > 3-HPA.[27-29] This hardness order is consistent with our MALDI-TOF MS results. For other peptides examined in this study, the same trend was also observed (spectra now shown).

It is also notable that the abundance of unconjugated peptide (RVYIHPFHL) was negligible with 3-HPA (see **Figure 1f**), while significant with CHCA, DHB, and *super* DHB. For THAP and dithranol, an unassignable peak at *m/z* 1148.5 (but presumably related to the conjugated peptide) occurs in a high abundance. It appears that 3-HPA selectively suppressed the generation of the unconjugated peptide and the fragmentation of the conjugated peptide, thus making itself an attractive matrix for MALDI-TOF/TOF MS studies. Therefore, in the following tandem mass spectrometry studies, only 3-HPA was used as a MALDI matrix.

MALDI-TOF/TOF MS/MS

Figure 2 shows the MALDI-TOF/TOF MS/MS spectra obtained for (a) the intact and (b) *p*-TEMPO-Bz-Sc-ARVYIHP. At a glance, the two MS/MS spectra appear to be very similar to each other. In both spectra, ***a***-type fragments were major products and ***b***-/***y***-type ions were minor. However, a closer look at the results reveals a few differences. First, there were many unassigned abundant peaks in the low *m/z* region of the TOF/TOF spectrum for the intact ARVYIHP. In contrast, these unassigned peaks in the low *m/z* region significantly reduced or disappeared in the spectrum for *p*-TEMPO-Bz-Sc-ARVYIHP. Most of the

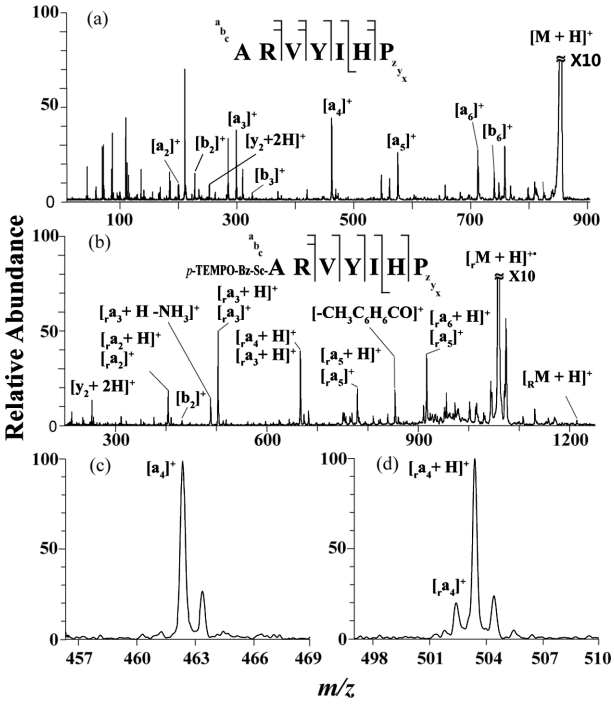


Figure 2. MALDI-TOF MS/MS spectra of (a) intact ARVYIHP and (b) *p*-TEMPO-Bz-Sc-conjugated ARVYIHP acquired on an ABI 4800 plus MALDI-TOF/TOF analyzer (air was used as a collision gas). (c) and (d) are the enlarged MS/MS spectrum of (a) and (b), respectively.

peaks in **Figure 2b** were assigned as sequence or side-chain loss peaks. Second, side-chain loss peaks were found more extensively in the *p*-TEMPO-Bz-Sc-conjugated peptide. More importantly, $[a_n]^+$ ions were generated as ***a***-type ions for the intact peptide, but $[a_n]^+$ and $[a_n+H]^+$ ions were identified as a pair in the spectrum of the *p*-TEMPO-Bz-Sc-conjugated peptide (see **Figure 2 c** and **d**, enlarged spectra). The relative abundances of the observed $[a_n]^+$ fragments were much lower than those of the $[a_n+H]^+$ ions in the *p*-TEMPO-Bz-Sc-conjugated peptide spectrum. The simultaneous occurrences of $[a_n]^+$ and $[a_n+H]^+$ as a pair were observed also in the MALDI-TOF/TOF MS/MS spectra for *p*-TEMPO-Bz-Sc-RVYIHPFHL (see Supplementary materials, **Figure S1**). However, for *p*-TEMPO-Bz-Sc-DRVYIHPFHLLVYS, which is the longest peptide examined in this study, only $[a_n+H]^+$ ions were observed, except for $[a_8]^+$ (see Supplementary materials, **Figure S2 (b)**).

The major occurrence of ***a***-type ions in MALDI-TOF/TOF intact peptide collision experiments originates from high kinetic energy collisions between the intact peptide ions and air or argon collision gases. Indeed, in previous MALDI-TOF/TOF tandem mass spectrometry studies, it was shown that ***a***-type fragment ions and side-chain loss peaks were major products when argon was used as a collision gas.[9,10,30] Furthermore, sector tandem mass spectrometry, which uses several keV for collision energy, has a similar fragmentation pattern for ***a***-type fragment production.[31] In contrast, the $[a_n+H]^+$ ions observed for the *p*-TEMPO-Bz-Sc-conjugated peptides are very likely to derive from fragmentation mechanisms that are quite different from that of $[a_n]^+$ observed for the intact peptides. In the MALDI-TOF/TOF MS/MS *p*-TEMPO-Bz-Sc-conjugated peptide spectra, $[a_n+H]^+$ type fragments were observed as a major product paring with low abundance $[a_n]^+$, in contrast to $[a_n]^+$ in the spectra for intact peptides. The occurrence of $[a_n+H]^+$ can be explained based on the radical-mediated mechanism, which occurs via β-fragmentation at the β-carbon radical center. This mechanism was described in a previous study.[13] Conversely, the major yield of $[a_n+H]^+$ type products implies that the radical-driven fragmentation mechanism was at play in peptide tandem mass spectrometry.

Peptides with arginine in the C-terminal region

The peptides described above all contain a basic arginine residue, which is known to have the highest proton affinity, in the N-terminal region. Therefore, we examined peptides with arginine in the C-terminal region. The MALDI-TOF/TOF spectrum for the (conjugated and unconjugated) peptide ALPMHIR was acquired using a Bruker UltrafleXtreme MALDI-TOF/TOF mass spectrometer. This instrument uses a LIFT post-source decay method for tandem mass spectrometry technique. In contrast to those with an arginine in the N-terminal region, the conjugated ALPMHIR had several C-terminal products, *e.g.*, ***x***-, ***y***-, and ***z***-type fragments as well as some ***a***- and ***b***-type N-terminal products (see **Figure S3**). Except for this, this tandem mass spectrometry spectrum did not show any noticeable spectral features that are worth mentioning in detail.

Conclusions

In this study, a MALDI ionization method was applied to the TEMPO-assisted FRIPS MS. The performance of six different matrices was evaluated and compared with the other matrices used in previous MALDI-TOF(/TOF) MS studies. We found that 3-HPA is the most optimal matrix for this purpose. Similar to ESI-(LC)-MS/MS, MALDI-TOF/TOF MS for the *p*-TEMPO-Bz-Sc-conjugated peptides produced extensive peptide fragments, which indicates that MALDI-TOF/TOF is an alternative tandem mass spectrometry tool for TEMPO-assisted FRIPS MS.

Supporting Information

Supplementary information is available at https://drive.google.com/file/d/14WQBeAYmtHJZekd0OI7nWZlJ17aPilHl/view?usp=sharing.

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References

1. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, 246, 64.
2. Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. *Anal. Chem.* **1991**, 63, 1193.
3. Pieles, U.; Zürcher, W.; Schär, M.; Moser, H. E. *Nucleic Acid Res.* **1993**, 21, 3191.
4. Fenn, J. B. *J. Biomol. Tech.* **2002**, 13, 101.
5. Spengler, B.; Kirsch, D.; Kaufmann, R.; Jaeger, E. *Rapid Commun. Mass Spectrom.* **1992**, 6, 105.
6. Ji, J.; Chakraborty, A.; Geng, M.; Zhang, X.; Amini, A.; Bina, M.; Regnier, F. *J. Chromatogr. B* **2000**, 745, 197.
7. Fenselau, C.; Demlrev, P. A. *Mass Spectrom. Rev.* **2001**, 20, 157.
8. Singhal, N.; Kumar, M.; Kanaujia, P. K.; Virdi, J. S. *Front. Microbiol.* **2015**, 6.
9. Medzihradszky, K. F.; Campbell, J. M.; Baldwin, M. A.; Falick, A. M.; Juhasz, P.; Vestal, M. L.; Burlingame, A. L. *Anal. Chem.* **2000**, 72, 552.
10. Suckau, D.; Resemann, A.; Schuerenberg, M.; Hufnagel, P.; Franzen, J.; Holle, A. *Anal. Bioanal. Chem.* **2003**, 376, 952.

11. Zubarev, R. A. *Curr. Opin. Biotechnol.* **2004**, 15, 12.
12. Syka, J. E. P.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, 101, 9528.
13. Lee, M.; Kang, M.; Moon, B.; Oh, H. B. *Analyst* **2009**, 134, 1706.
14. Lee, M.; Lee, Y.; Kang, M.; Park, H.; Seong, Y.; Sung, B. J.; Moon, B.; Oh, H. B. *J. Mass Spectrom.* **2011**, 46, 830.
15. Jeon, A.; Lee, J. H.; Kwon, H. S.; Park, H. S.; Moon, B.; Oh, H. B. *Mass Spectrom. Lett.* **2013**, 4, 71.
16. Lee, J.; Park, H.; Kwon, H.; Kwon, G.; Jeon, A.; Kim, H. I.; Sung, B. J.; Moon, B.; Oh, H. B. *Anal. Chem.* **2013**, 85, 7044.
17. Kwon, G.; Kwon, H.; Lee, J.; Han, S. Y.; Moon, B.; Oh, H. B.; Sung, B. J. *Bull. Kor. Chem. Soc.* **2014**, 35, 700.
18. Marshall, D. L.; Hansen, C. S.; Trevitt, A. J.; Oh, H. B.; Blanksby, S. J. *Phys. Chem. Chem. Phys.* **2014**, 16, 4871.
19. Jeon , A.; Hwangbo, S.; Ryu, E. S.; Lee, J.; Yun, K. N.; Kim, J. Y.; Moon, B.; Oh, H. B. *Int. J. Mass Spectrom.* **2015**, 390, 110.
20. Oh, H. B.; Moon, B. *Mass Spectrom. Rev.* **2015**, 34, 116.
21. Nam, J.; Kwon, H.; Jang, I.; Jeon, A.; Moon, J.; Lee, S. Y.; Kang, D.; Han, S. Y.; Moon, B.; Oh, H. B. *J. Mass Spectrom.* **2015**, 50, 378.
22. Lee, C. S.; Jang, I.; Hwangbo, S.; Moon, B.; Oh, H. B. *Bull. Kor. Chem. Soc.* **2015**, 36, 810.
23. Jang, I.; Lee, S. Y.; Hwangbo, S.; Kang, D.; Lee, H.; Kim, H. I.; Moon, B.; Oh, H. B. *J. Am. Soc. Mass Spectrom.* **2017**, 28, 154.
24. Zubarev, R. A.; Kruger, N. A.; Fridriksson, E. K.; Lewis, M. A.; Horn, D. M.; Carpenter, B. K.; McLafferty, F. W. *J. Am. Chem. Soc.* **1999**, 121, 2857.
25. Shi, S. D. H.; Hemling, M. E.; Carr, S. A. *Anal. Chem.* **2001**, 73, 19.
26. Molina, H.; Horn, D. M.; Tang, N.; Mathivanan, S.; Pandey, A. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, 104, 2199.
27. Vertes, A.; Luo, G.; Marginea, L. *Anal. Chem.* **2002**, 74, 6185.
28. Gabelica, V.; Schulz, E.; Karas, M. *J. Mass Spectrom.* **2004**, 39, 579.
29. Bae, Y. J.; Moon, J. H.; Kim, M. S. *J. Am. Soc. Mass Spectrom.* **2011**, 22, 1070.
30. Kaufmann, R.; Spengler, B.; Lutzenkirchen, F. *Rapid Commun. Mass Spectrom.* **1993**, 7, 902.
31. Bordas-Nagy, J.; Despeyroux, D.; Jennings, K. R. *J. Am. Soc. Mass Spectrom.* **1992**, 3, 502.

