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RESEARCH ARTICLE

$C_\alpha - C$ Bond Cleavage of the Peptide Backbone in MALDI In-Source Decay Using Salicylic Acid Derivative Matrices

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Abstract

The use of 5-formylsalicylic acid (5-FSA) and 5-nitrosalicylic acid (5-NSA) as novel matrices for in-source decay (ISD) of peptides in matrix-assisted laser desorption/ionization (MALDI) is described. The use of 5-FSA and 5-NSA generated *a*- and *x*-series ions accompanied by oxidized peptides $[M - 2\,H + H]^+$. The preferential formation of *a*- and *x*-series ions was found to be dependent on the hydrogen-accepting ability of matrix. The hydrogen-accepting ability estimated from the ratio of signal intensity of oxidized product $[M - 2\,H + H]^+$ to that of non-oxidized protonated molecule $[M + H]^+$ of peptide was of the order 5-NSA>5-FSA>5-aminosalicylic acid (5-ASA) ≒ 2,5-dihydroxyl benzoic acid (2,5-DHB) ≒ 0. The results suggest that the hydrogen transfer reaction from peptide to 5-FSA and 5-NSA occurs during the MALDI-ISD processes. The hydrogen abstraction from peptides results in the formation of oxidized peptides containing a radical site on the amide nitrogen with subsequent radical-induced cleavage at the $C_\alpha - C$ bond, leading to the formation of *a*- and *x*-series ions. The most significant feature of MALDI-ISD with 5-FSA and 5-NSA is the specific cleavage of the $C_\alpha - C$ bond of the peptide backbone without degradation of side-chain and post-translational modifications (PTM). The matrix provides a useful complementary method to conventional MALDI-ISD for amino acid sequencing and site localization of PTMs in peptides.

Key words: Hydrogen-accepting matrix, Matrix-assisted laser desorption/ionization, In-source decay, Phosphorylated peptides

Introduction

Mass spectrometry has been used as a powerful analytical tool in a wide variety of scientific fields because of its high sensitivity and rapidity. Of the soft ionization methods, matrix-assisted laser desorption/ionization (MALDI) [1–3] and electrospray ionization (ESI) [4, 5] are recognized as indispensable analytical methods for identifying biological polymers such as proteins, nucleic acids, and oligosaccharides. In particular, peptide-mass fingerprinting (PMF) [6, 7] and amino acid sequencing with tandem mass spectrometry [8, 9] have become common approaches for the characterization of proteins.

In MALDI, a prompt fragmentation named "in-source decay (ISD)" occurs in the ion source before the ion extraction. MALDI-ISD is initiated by the transfer of a hydrogen atom from an excited matrix molecule to the carbonyl group of a peptide backbone, leading to a hydrogen-abundant peptide [10, 11]. Subsequently, the $N - C_\alpha$ bond on the peptide backbone is cleaved, leading to the formation of *c*′- and *z*′-series ions (Scheme 1a). The hydrogen atoms in MALDI-ISD are produced from the active hydrogens of matrix molecules such as the 5-hydroxyl group in 2,5-dihydroxybenzoic

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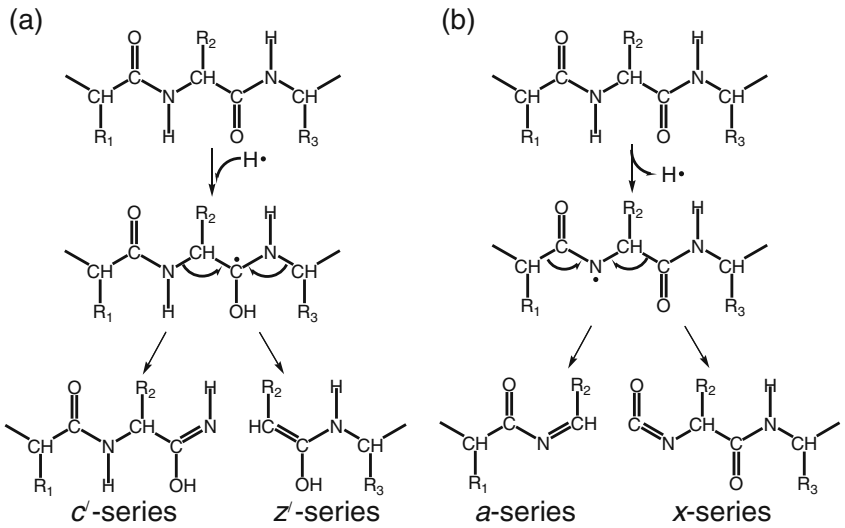


Scheme **1**. The mechanisms of radical-induced fragmentation via hydrogen attachment (a) and hydrogen abstraction (b)

acid (2,5-DHB) [10], the amino group in 1,5-diaminonaphtalene (1,5-DAN) [12], and the 5-amino group in 5-aminosalicylic acid (5-ASA) [13], which are activated by the irradiation of UV-laser photons. It is important to recognize that hydrogen-donating ability is a prominent factor for the function of matrix in MALDI-ISD [12–14]. The hydrogen-donating ability can be measured by studying the ability to cleave disulfide bonds by reduction [12–14]. The order of hydrogen-donating ability is 1,5-DAN>5-ASA>2,5-DHB > α-cyano-4-hydroxycinnamic acid (CHCA) [13, 14]. In particular, 5-ASA gave peaks with greater sharpness for protonated molecules and fragment ions than other matrices and did not give any interference peaks such as metastable ions and/or matrix clusters [13].

MALDI-ISD shares some similarities with radical-induced fragmentation, electron capture dissociation (ECD) [15], and electron transfer dissociation (ETD) [16]. Both ECD and ETD involve attaching electrons to multiply protonated analytes $[M + nH]^{n+}$. A proton bound to an analyte is converted into a hydrogen atom via electron attachment and the hydrogen atom can transfer to the carbonyl oxygen on the peptide backbone with subsequent radical-induced cleavage at the $N - C_\alpha$ bonds [17]. As a result, ECD and ETD give c'- and z'-series ions. The most significant feature of MALDI-ISD and ECD/ETD is the specific cleavage of the $N - C_\alpha$ bond of the peptide backbone without degradation of side chain. Therefore, MALDI-ISD and ECD/ETD have been utilized as tools for characterization of post-translational modifications and a top-down approach to protein identification [18–20]. Recently, new radical-induced fragmentation methods such as electron detachment dissociation (EDD) [21] and negative electron transfer dissociation (NETD) [22] have been developed for identifying negative ion peptides. Both EDD and NETD involve detaching electrons from multiply deprotonated analytes $[M - nH]^{n-}$. Electron detachment results in the formation of a charge-reduced peptide anion that contains a radical site on the carboxyl group of the side chain or C-terminal carboxyl group. Subsequently, a nitrogen-centered radical product is formed via hydrogen transfer from the backbone amide nitrogen to the radical site on the carboxyl group. The radical on the amide nitrogen induces dissociation of the $C_\alpha - C$ bond, leading to the formation of *a*- and *x*-series ions without degradation of the side chain (Scheme 1b) [23].

Here we report that the use of oxidizing matrices such as 5-nitrosalicylic acid (5-NSA) and 5-formylsalicylic acid (5-FSA) result in selective cleavage at the $C_\alpha - C$ bond of the peptide backbone, leading to the formation of *a*- and *x*-series ions. Positive ion MALDI-ISD with 5-FSA and 5-NSA shares some similarities with EDD and NECD. The mechanism of the cleavage of the $C_\alpha - C$ bond on peptide backbone in MALDI-ISD is described.

Experimental

Materials

All peptides were purchased from Peptide Institute (Osaka, Japan). The sequences of peptides used are summarized in Table 1. The MALDI matrices, 2,5-dihydroxyl benzoic acid

Table 1. Monoisotopic Mass (M_m) and Sequence of Analyte Peptides Used

Analyte peptide	M_m	Sequence
ACTH18-35	1977.95	RPVKVYPNGAEDESAEAF
[pTyr^6]-ACTH18-35	2057.93	RPVKVpYPNGAEDESAEAF
[pTyr^6, pSer^{14}]-ACTH18-35	2137.91	RPVKVpYPNGAEDEpSAEAF
[Arg^{18}]-ACTH19-36	1977.95	PVKVYPNGAEDESAEAFR
Synthetic peptide	1427.72	RLGNQWAVGDLAE
Synthetic deuterium-labeled peptide	1433.72	RLGNQWA(d_3)VG(d_2)DLAE
[Arg^8]-vasopressin	1083.44	CYFQNCPRG(NH_2)
Substance P	1346.72	RPKPQQFFGLM(NH_2)

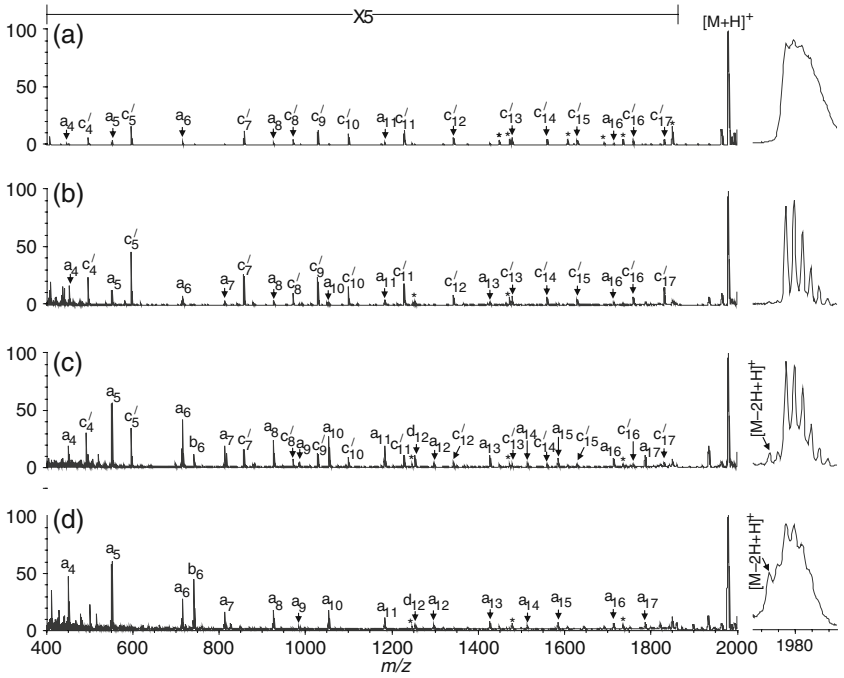


Figure 1. Positive-ion MALDI-ISD spectra of ACTH18-35 obtained with **(a)** 2,5-DHB, **(b)** 5-ASA, **(c)** 5-FSA, and **(d)** 5-NSA. Asterisk indicates metastable peaks. The right hand inset represents the peaks of protonated molecule $[M+H]^+$

(2,5-DHB), 5-amino salicylic acid (5-ASA), 5-formyl salicylic acid (5-FSA), and 5-nitro salicylic acid (5-NSA) were purchased from Tokyo Kasei (Tokyo, Japan). Trifluoroacetic acid (TFA) and acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). Water used in all experiments was purified using a MilliQ water purification system from Millipore (Billerica, MA, USA). All reagents were used without further purification.

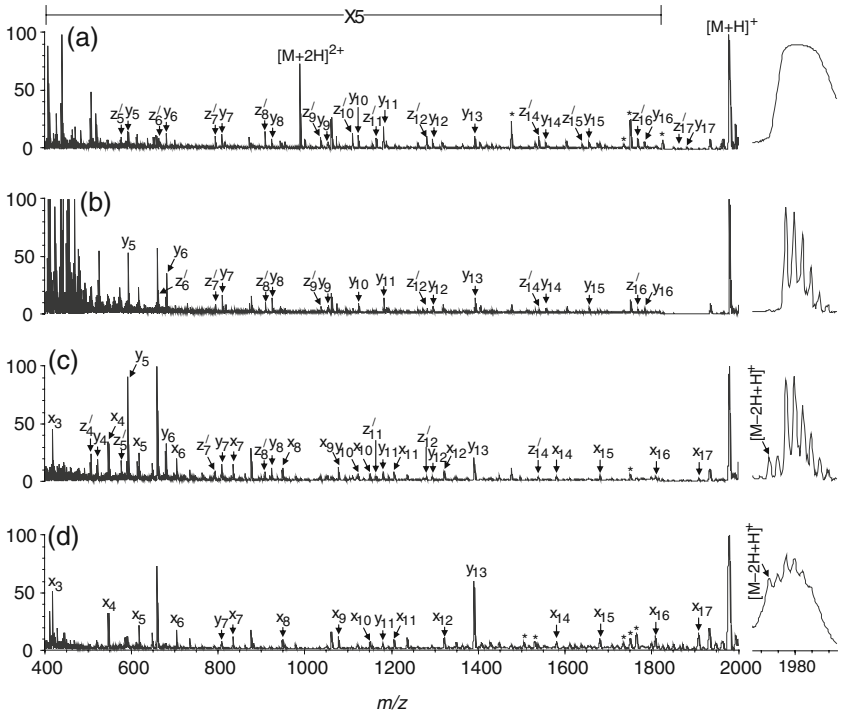


Figure 2. Positive-ion MALDI-ISD spectra of [Arg^{18}]-ACTH18-35 obtained with **(a)** 2,5-DHB, **(b)** 5-ASA, **(c)** 5-FSA, and **(d)** 5-NSA. Asterisk indicates metastable peaks. The right hand inset represents the peaks of protonated molecule $[M+H]^+$

Table 2. Observed Fragment Ions in the MALDI-ISD Spectra Obtained with 2,5-DHB, 5-ASA, 5-FSA, and 5-NSA

Analyte peptide	Matrix	Observed ISD fragment ions
ACTH18-35	2,5-DHB	R P V K V Y P N G A E D E S A E A F
	5-ASA	R P V K V Y P N G A E D E S A E A F
	5-FSA	R P V K V Y P N G A E D E S A E A F
	5-NSA	R P V K V Y P N G A E D E S A E A F
[Arg^{18}]-ACTH19-36	2,5-DHB	P V K V Y P N G A E D E S A E A F R
	5-ASA	P V K V Y P N G A E D E S A E A F R
	5-FSA	P V K V Y P N G A E D E S A E A F R
	5-NSA	P V K V Y P N G A E D E S A E A F R

Sample Preparation

Analyte peptides were dissolved in water at a concentration of 20 pmol/μL. 2,5-DHB and 5-NSA were dissolved in water/acetonitrile (1:1, vol/vol) with 0.1% TFA at a concentration of 10 mg/mL. 5-ASA was dissolved in water/acetonitrile (1:1, vol/vol) with 0.3% TFA at a concentration of 5 mg/mL; 5 μL of analyte solution was mixed with 5 μL of matrix solution. A volume of 1 μL of sample solution was deposited onto a stainless steel plate and the solvents were removed by allowing evaporation in air at room temperature; 5-FSA was dissolved in acetone at a concentration of 10 mg/mL. A volume of 0.5 μL of analyte solution was deposited onto a stainless-steel MALDI target and left to dry. After complete evaporation of the solvent, 0.5 μL of matrix solution in acetone was deposited onto the dried peptides.

Matrix-Assisted Laser Desorption /Ionization Mass Spectrometry

MALDI mass spectra were obtained using a time-of-flight mass spectrometer, AXIMA-CFR (Shimadzu, Kyoto, Japan) equipped with a nitrogen laser (337 nm wavelength) operating at a pulse rate of 10 Hz. The pulse width of the laser was 4 ns. The laser spot size on the target substrate was about 100 μm in diameter. The appropriate laser fluences (μJ/pulse) for the MALDI-ISD were about 6.5 for 2,5-DHB and 5-NSA, and 18.9 for 5-ASA and 5-FSA. The ions generated by MALDI were accelerated using 20 kV with delayed extraction. The analyzer was operated in reflectron mode and the ions were detected using a microchannel plate detector. A total of 500 shots were accumulated for each mass spectrum acquisition.

Results and Discussion

Matrix Effect on the In-Source Decay Fragment Ions: Hydrogen-Accepting Nature of Matrix

Here we employ Zubarev's notation for the ISD fragment ions [24]. According to the notation, homolytic $C_\alpha - C$ bond cleavage gives $a\cdot$ and $x\cdot$ fragments, and hydrogen atom loss from $a\cdot$ and $x\cdot$ fragments gives a and x fragments, respectively. In contrast, hydrogen atom transfer to a and x fragments is denoted by a' and x', respectively. Thus, the a and x fragments are 1.0078 Da smaller than the $a\cdot$ and $x\cdot$ fragments, respectively, and the a' and x' fragments are 1.0078 Da larger than the $a\cdot$ and $x\cdot$ fragments.

Figure 1 shows the comparison of positive-ion MALDI mass spectra of adrenocorticotropic hormone fragment 18–35 (ACTH18-35) obtained with four different matrices 2,5-DHB, 5-ASA, 5-FSA and 5-NSA. The ISD fragment ions were observed in all mass spectra. The use of 2,5-DHB and 5-ASA generated c'-series ions accompanied by a-series ions with weak intensity. In MALDI-ISD, the peptides are principally cleaved at the $N - C_\alpha$ bond on the peptide backbone, giving c'- and z'-series ions. Subsequently, c'-series ions induce dissociation on the $C_\alpha - C$ bond, leading

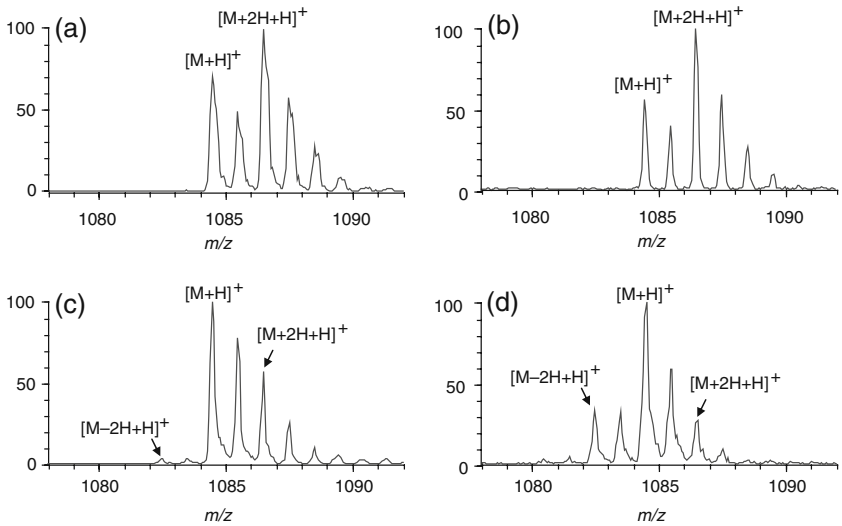


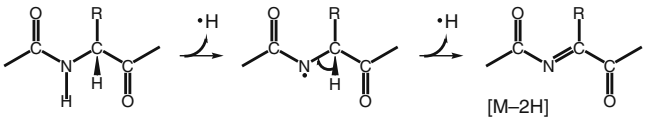
Figure 3. Partial MALDI mass spectra of [Arg^8]-vasopressin obtained with **(a)** 2,5-DHB, **(b)** 5-ASA, **(c)** 5-FSA, and **(d)** 5-NSA

to *a*-series ions with hydrogen transfer from the β-carbon [13]. In the MALDI mass spectra with 2,5-DHB and 5-ASA, the a_9 ion was not observed in the $C_\alpha - C$ bond cleavage at the Gly–Ala bond due to lack of a β-hydrogen in the Gly residue. The lack of an *a* ion at the Gly residue can be seen in the MALDI mass spectra reported previously [10, 13, 25].

Although the use of 5-FSA generated both *a*- and *c*′-series ions, the signal abundance of the *c*′-series ions was less intense than that of the *a*-series ions (Figure 1c). Additionally, the a_9 ion originating from the cleavage of the $C_\alpha - C$ bond at Gly–Ala was observed. This indicates that the *a*-series ions are generated without hydrogen transfer from the β-carbon by using 5-FSA as a matrix. This suggests that the formation of the *a*-series ions with 5-FSA is via a different in mechanism from that when conventional matrices 2,5-DHB and 5-ASA are used. In contrast, the *c*′-series ions originate from the cleavage at the $N - C_\alpha$ bond by intermolecular hydrogen transfer from the formyl hydrogen of 5-FSA to the peptide backbone carbonyl-oxygen. It was previously known that the formyl group not only has hydrogen-donating nature but also hydrogen-accepting properties. Thus, the *a*-series ions originate from the hydrogen accepting nature of the formyl group in 5-FSA. A proposed pathway for the formation of the *a*-series ions in MALDI-ISD with 5-FSA is shown in Scheme 1b. Here we focus on the hydrogen-accepting nature of matrix.

It has previously been reported that the nitro group has a hydrogen-accepting nature [26]. It is expected that the presence of a nitro group in a matrix molecule would enhance the formation of *a*-series ions. To ascertain the formation mechanism of *a*-series ions, 5-nitrosalicilyc acid (5-NSA) was used as a MALDI matrix (Figure 1d). The use of 5-NSA did not generate any *c*′-series ions, and instead *a*-series ions were observed with strong signal intensities. The difference in the nature of functional groups at the 5-position in salicylic acid derivatives of the MALDI matrix can dramatically affect the ISD products. The same trend was obtained with another peptide, namely substance P (Supporting Information, Figure S1). The data suggest that the use of 5-FSA and 5-NSA leading to the formation of *a*-series ions accompanies hydrogen abstraction, as shown in Scheme 1b.

It was previously known that peptides containing basic amino acid residues, especially an Arg residue, near the N-terminus, preferentially gave *c*′- and *a*-series ions, while the presence of basic residues at the C-terminal favored the formation of *y*- and *z*′-series ions [27]. A peptide [Arg^{18}]-ACTH19-36 having an Arg residue at the C-terminus would be expected to give *x*-, *y*- and/or *z*′-series ions in ISD experiments. Figure 2 shows the comparison of positive-ion MALDI-ISD spectra of [Arg^{18}]-ACTH19-36 obtained with four different matrices 2,5-DHB, 5-ASA, 5-FSA and 5-NSA. All the spectra show *x*-, *y*- and/or *z*′-series ions. The MALDI-ISD spectra with 2,5-DHB and 5-ASA exclusively showed *y*- and *z*′-series ions (Figure 2a and b). In contrast, *x*-, *y*- and *z*′-series ions were observed in MALDI-ISD spectra with 5-FSA (Figure 2c), whereas the use of 5-NSA did not show *z*′-series ions but the *x*- and *y*-series ions were observed (Figure 2d). It is interesting that 5-FSA and 5-NSA give principally the *a*-series ions of ACTH18-35 and *x*-series ions of [Arg^{18}]-ACTH19-36 as this indicates cleavage at the $C_\alpha - C$ bonds of the peptide backbone. The fragment ions observed in the MALDI-ISD spectra with 2,5-DHB, 5-ASA, 5-FSA and 5-NSA are summarized in Table 2. The cleavage at the $C_\alpha - C$ bond occurs with hydrogen abstraction from peptides to matrix molecules, as shown in Scheme 1b. Specific cleavage at $C_\alpha - C$ bonds is observed to yield *a*/*x*-series ions due to the hydrogen-accepting nature of the nitro group of 5-NSA. In contrast, the use of 5-FSA results in cleavage at both $N - C_\alpha$ and $C_\alpha - C$ bonds because 5-FSA has both hydrogen-donating and hydrogen-accepting properties.



Scheme **2**. Mechanism for the formation of [M – 2H]

Hydrogen-Donating and Hydrogen-Accepting Ability of Salicylic Acid Derivatives

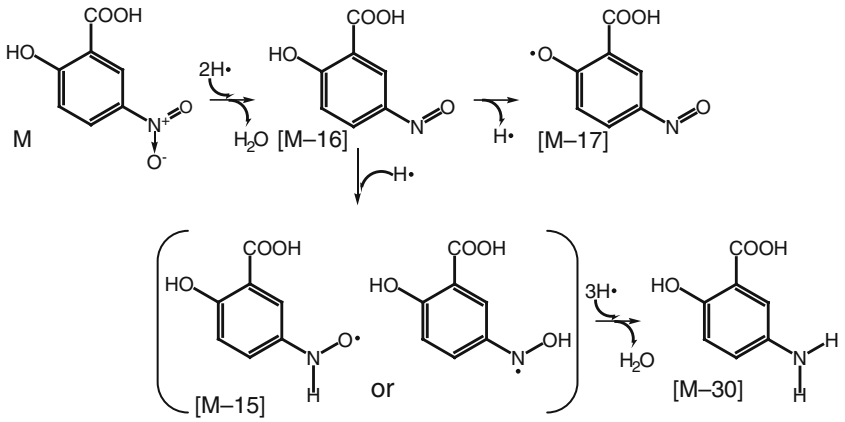
It is important to recognize that both hydrogen-donating and -accepting properties are important factors in the function of matrix in MALDI-ISD. The hydrogen-donating ability can be estimated by measuring the ability to reduce disulfide bonds (S–S) [12–14]. Figure 3 shows positive-ion MALDI mass spectra of the molecular-related ions of [Arg^8]-vasopressin, which contains a disulfide bond between Cys^1 and Cys^6, obtained with four different matrices, 2,5-DHB, 5-ASA, 5-FSA ,and 5-NSA. The use of 2,5-DHB and 5-ASA gave high ion yields of the reduced ion $[M+2\ H + H]^+$, while the use of 5-FSA and 5-NSA gave medium or low ion yields of $[M+2\ H + H]^+$. The hydrogen-donating ability (%) estimated from the ratio of signal intensity of the reduced ion $[M+2\ H + H]^+$ to that of the non-reduced ion $[M + H]^+$ of [Arg^8]-vasopressin is summarized in Table 3. The

Table 3. Hydrogen-Donating Ability of Salicylic Acid Derivatives

	2,5-DHB	5-ASA	5-FSA	5-NSA
[Arg^8]-vasopressin	80	110	35	10

Table 4. Hydrogen-Accepting Ability of Salicylic Acid Derivatives

	2,5-DHB	5-ASA	5-FSA	5-NSA
ACTH18-35	0	0	15	60
[Arg^{18}]-ACTH19-36	0	0	22	76
Synthetic peptide	0	0	13	61
Substance P	0	0	23	65
[Arg^8]-vasopressin	0	0	5	39



Scheme **3**. Redox reactions of 5-NSA

peak abundance of $[M+2\ H\ +\ H]^+$ was corrected by theoretical isotope abundance of $[M\ +\ H+2]^+$ for $[Arg^8]$-vasopressin. The presence of a 5-amino group in 5-ASA and a 5-hydroxyl group in 2,5-DHB seems to be advantageous for the intermolecular hydrogen transfer from the matrix to the analyte peptide. The order of the ascertained hydrogen-donating ability was 5-ASA>2,5-DHB>5-FSA>5-NSA.

In contrast, the use of 5-FSA and 5-NSA gave dehydrogenated or oxidized $[Arg^8]$-vasopressin $[M\ –\ 2\ H\ +\ H]^+$, as shown in Figure 3c and d. The oxidized product $[M\ –\ 2\ H\ +\ H]^+$ was also observed in the mass

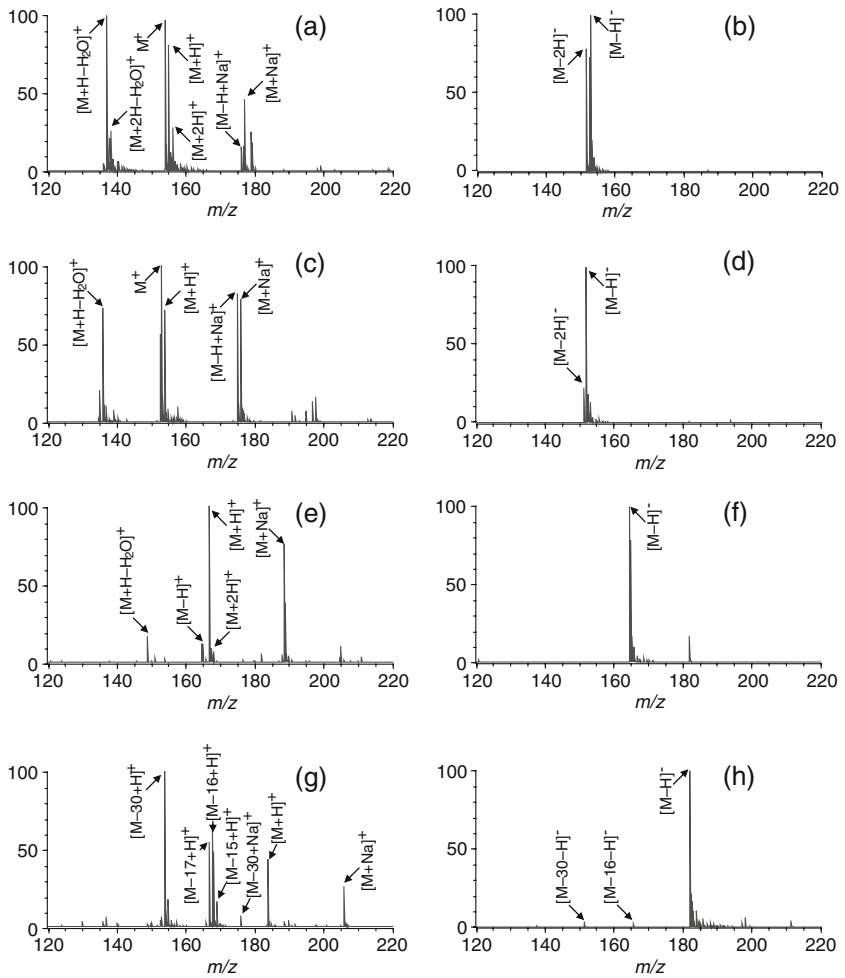


Figure 4. Mass spectra of 2,5-DHB **(a)** positive-ion mode, **(b)** negative-ion mode, 5-ASA **(c)** positive-ion mode, **(d)** negative-ion mode, 5-FSA **(e)** positive-ion mode, **(f)** negative-ion mode, and 5-NSA **(g)** positive ion mode, **(h)** negative ion mode

spectra of other peptides, as shown in the insets of Figures 1 and 2. The oxidized product $[M - 2\ H + H]^+$ was formed by hydrogen transfer from peptide molecules to the 5-formyl group in 5-FSA and the 5-nitro group in 5-NSA. A proposed pathway for the formation of $[M - 2\ H + H]^+$ is shown in Scheme 2. As described above, the amide hydrogen on the peptide backbone is the most probable candidate for the release of a hydrogen atom. Hydrogen abstraction from peptide results in the formation of a peptide radical where the radical site is on the amide nitrogen. Subsequently, the radical induces dissociation of the $C_\alpha - H$ bond, leading to the formation of $[M - 2\ H + H]^+$. The hydrogen-accepting ability (%) of the matrix estimated from the ratio of signal intensity of oxidized ion $[M + H - 2\ H]^+$ to that of non-oxidized ion $[M + H]^+$ of peptides is summarized in Table 4. The order of the ascertained hydrogen-accepting ability was 5-NSA>5-FSA>5-ASA ≒ 2,5-DHB ≒ 0. The use of 5-FSA and 5-NSA gave moderate or intense *a*- and *x*-series ions in MALDI-ISD experiments. The abundances of *a*-series ions in Figure 1 and *x*-series ions in Figure 2 were dependent on the hydrogen-accepting ability of matrix.

Redox Products of Matrix Molecules

The use of 2,5-DHB and 5-ASA gave a large abundance of reduced $[Arg^8]$-vasopressin $[M+2\ H+H]^+$, as seen in Figure 3. This suggests that the oxidative reaction of 2,5-DHB and 5-ASA molecules occurs in the MALDI process. Figure 4 shows positive- and negative-ion MALDI mass spectra of the four different matrices, 2,5-DHB, 5-ASA, 5-FSA, and 5-NSA. All the spectra show the molecular-related signals $[M + H]^+$, $[M + Na]^+$, and $[M - H]^-$. The oxidized products of matrix, $[M - H + Na]^{+\cdot}$ and $[M - 2\ H]^{-\cdot}$, were observed in the mass spectra of 2,5-DHB and 5-ASA, while those products were almost absent from the mass spectra of 5-FSA and 5-NSA. These products were formed by releasing a hydrogen atom from the 5-hydroxy group of 2,5-DHB and the 5-amino group of 5-ASA. In contrast, the reduced matrix products $[M + 2\ H]^{+\cdot}$ and $[M + 2\ H - H_2O]^{+\cdot}$ were observed in the MALDI mass spectra of 2,5-DHB, but the use of 2,5-DHB did not generate the oxidized peptides $[M - 2\ H + H]^+$. This suggests that the hydrogen transfer reaction from peptide to 2,5-DHB did not occur in the MALDI-ISD processes. Therefore, the reduction products of 2,5-DHB, $[M + 2\ H]^{+\cdot}$ and $[M + 2\ H - H_2O]^{+\cdot}$, are formed by a hydrogen transfer reaction from 2,5-DHB to other 2,5-DHB molecules.

The positive-ion MALDI mass spectrum of 5-FSA shows an oxidized ion $[M - H]^+$ originating from the release of hydrogen from the formyl group in the molecular ion $M^{+\cdot}$ and reduced ion $[M+2\ H]^+$ of 5-FSA. The carbonyl oxygen in the formyl group is the most probable candidate for the capture of a hydrogen atom.

The use of 5-NSA gave an abundant peak of oxidized $[Arg^8]$-vasopressin $[M - 2\ H + H]^+$, formed by hydrogen transfer from $[Arg^8]$-vasopressin to 5-NSA. The mass spectra of 5-NSA show molecular-related signals $[M + H]^+$, $[M + Na]^+$, and $[M - H]^-$. Other product signals of $[M - 15+H]^+$, $[M - 16+H]^+$, $[M - 17+H]^+$, $[M - 30+H]^+$, and $[M - 30+Na]^+$ in Figure 5g and $[M - 16 - H]^-$ and $[M - 30 - H]^-$ in Figure 5h were also observed. It was previously known that the reduction of a nitro group $–NO_2$ leads to the formation of an amino group $–NH_2$. This reaction proceeds through intermediate stages involving a nitroso group –NO and a hydroxylamine group –NHOH [28]. It has also been reported that the nitro group moiety is partially converted into a nitroso group and an amino group by hydrogen transfer reactions during the MALDI process [29, 30]. The reduction reactions of 5-NSA are shown in Scheme 3. The loss of an oxygen atom from the nitro group of 5-NSA to form the reduced product [M – 16] containing a nitroso group –NO is involved in two hydrogen reduction process and is accompanied by the loss of water. The radical species [M – 17] is formed by a hydrogen transfer reaction from [M – 16] to other 5-NSA molecules. The reduced product [M – 30] containing an amino group $–NH_2$ is formed from further reduction reactions of [M – 16] via transfer of four hydrogen atoms. The mass spectra shown in Figure 4 suggest that oxidized peptides and reduced 5-NSA are formed by hydrogen transfer reaction from peptides to 5-NSA.

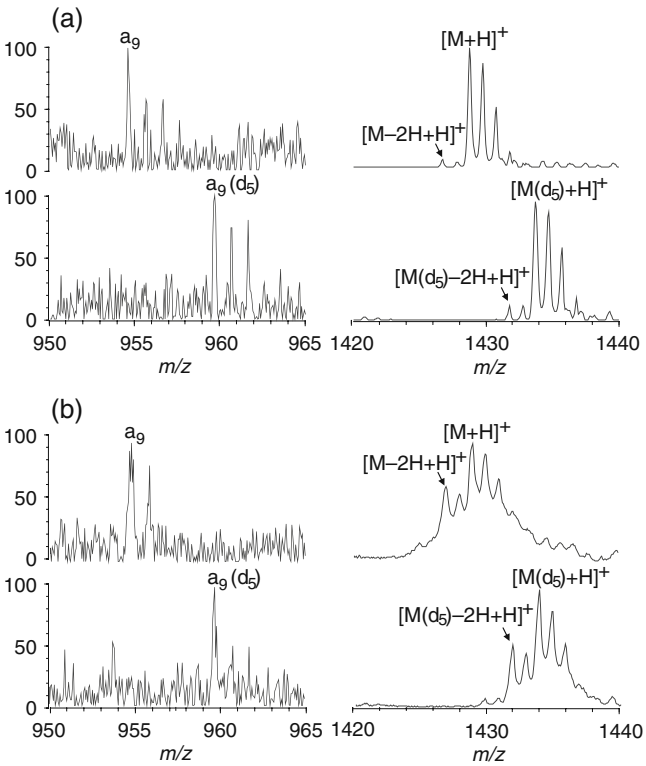
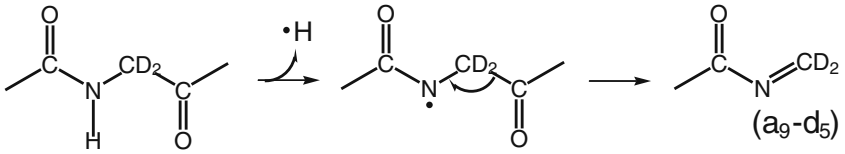


Figure 5. Partial MALDI mass spectra of synthetic peptide (upper panel) and synthetic deuterium-labeled peptide (lower panel) obtained with **(a)** 5-FSA and **(b)** 5-NSA



Scheme **4**. Mechanism for the formation of (a_9-d_5) ion

Abstraction of Amide Hydrogen Leads to $C_\alpha - C$ Bond Cleavage

MALDI mass spectra of a synthetic peptide RLGNQ WAVGDLAE, obtained with four different matrices are shown in Figure S2 (Supporting Information). The ISD fragment ions observed in all mass spectra showed a similar trend to those obtained for ACTH18-35 (Figure 1). To ascertain the most probable pathway for the formation of *a*- and *x*-series ions, a synthetic deuterium-labeled peptide RLGNQWA(d_3)VG(d_2)DLAE was used for MALDI-ISD with 5-FSA and 5-NSA. The deuterium labeled peptide contains Ala7 ($C_\beta D_3$) and Gly9 ($C_\alpha D_2$). The use of 5-FSA and 5-NSA resulted exclusively in the *a*-series ions for both synthetic peptides. The *a*-series ions in the former peptide were observed at *m/z* 413.2 (a_4), 541.3 (a_5), 727.4 (a_6), 798.4 (a_7), 897.5 (a_8), 954.5 (a_9), 1069.5 (a_{10}), 1182.6 (a_{11}), and 1253.6 (a_{12}), whereas deuterium-labeled peptide gave the *a*-series ions at *m/z* 413.2 (a_4), 541.3 (a_5), 727.4 (a_6), 801.4 (a_7-d_3), 900.5 (a_8-d_3), 959.5 (a_9-d_5), 1074.5 (a_{10}-d_5), 1187.6 (a_{11}-d_5), and 1258.6 (a_{12}-d_5). Enlarged spectra for a_9 and $[M + H]^+$ of these synthetic peptides are shown in Figure 5. A 5 Da mass shift was observed in the a_9 products. The mass shift is in agreement with the number of deuterium labels in the Ala7 ($C_\beta D_3$) and Gly9 ($C_\alpha D_2$). The a_9-d_4 product originating from deuterium abstraction from the Gly9 ($C_\alpha D_2$) carbon was not observed. This indicates that the *a*-series ions in the ISD spectra with 5-FSA and 5-NSA form via abstraction of the amide hydrogen on the peptide backbone, as shown in Scheme 4.

MALDI Mass Spectra of Phosphorylated Peptides with 5-FSA and 5-NSA

We have demonstrated the application of MALDI-ISD with 5-FSA and 5-NSA in the analysis of phosphopeptides. The MALDI mass spectra of mono-phosphorylated

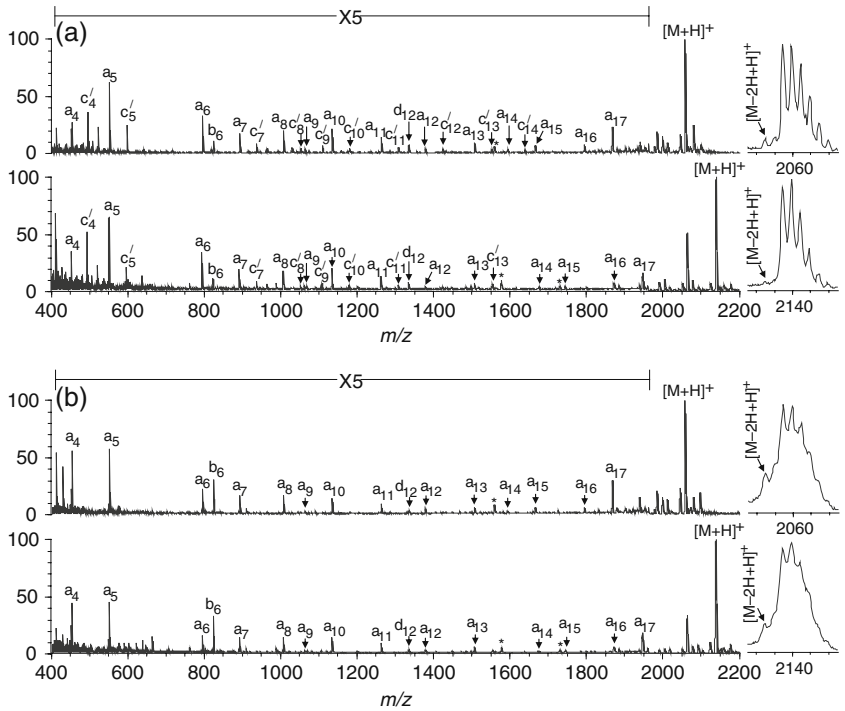


Figure 6. Positive-ion MALDI-ISND spectra of mono-phosphorylated peptide [pTyr6]-ACTH18-35 (upper panel) and di-phophorylated peptide [pTyr6, pSer14]-ACTH18-35 (lower panel) obtained with **(a)** 5-FSA and **(b)** 5-NSA. Asterisk indicates metastable peaks

peptide [pTyr^6]-ACTH18-35 and di-phosphorylated peptide [pTyr^6,pSer^{14}]-ACTH18-35 obtained using 5-FSA and 5-NSA are shown in Figure 6. The MALDI mass spectra of these peptides showed preferential formation of *a*-series ions from a_4 to a_{17}, although the use of 5-FSA generated *c*′-series ions as well. It is noteworthy that the loss or degradation of phosphoric group(s) in the phosphorylated peptides did not occur in the MALDI-ISD processes. The conservation of the phosphorylated site is crucial in terms of successfully identifying the sites of PTMs. MALDI-ISD of phosphorylated peptides with 5-ASA, 2,5-DHB, and 1,5-DAN have been reported previously [13]. The MALDI mass spectra of the phosphorylated peptides with 5-ASA showed *c*-series ions from c'_3 to c'_{17}, whereas several *c*′ ions in the ISD spectra with 2,5-DHB and 1,5-DAN were interfered with peak broadening, matrix clusters, and/or metastable peaks [13]. The use of 5-FSA and 5-NSA resulted in improved MALDI-ISD spectra of phosphorylated peptides over those observed using 2,5-DHB and 1,5-DAN, and compensate for the fragment ions of MALDI-ISD using 5-ASA.

Conclusions

MALDI-ISD of peptides has been studied using several salicylic acid derivatives as matrices. The difference in the nature of functional groups at the 5-position in the salicylic acid derivatives can dramatically affect the ISD products. The hydrogen-donating ability of matrix is a prominent factor in the generation of *c*′- and *z*′-series ions in MALDI-ISD. In contrast, hydrogen-accepting ability of matrix is found to be an important factor for the generation of *a*- and *x*-series ions. It is suggested that the nitrogen-centered radical can form by intermolecular hydrogen transfer from the peptide backbone amide hydrogen to the matrix with subsequent radical induced cleavage at the $C_\alpha - C$ bond. The MALDI with 5-FSA gave both *a*/*x* and *c*′/*z*′ fragment ions because 5-FSA has both hydrogen-donating and hydrogen-accepting properties. In contrast, the use of 5-NSA can result in selective cleavage at $C_\alpha - C$ bonds and lead to the formation of *a*/*x*-series ions due to the hydrogen-accepting nature. The use of 5-FSA and 5-NSA with mono- and di-phosphorylated peptides gave *a*-series ions without loss of phosphoric groups. The present matrix provides a useful complementary method to the conventional MALDI-ISD for the analysis of amino acid sequencing and site localization of PTMs in peptides.

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