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# Oxidative radical driven cleavage of peptide backbone caused by matrix-assisted laser desorption/ionization-in-source decay with low matrix-to-peptide molar ratios

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ABSTRACT

The influence of matrix-to-analyte molar ratio (*M/A*) on the formation of in-source decay (ISD) fragment ions was examined by matrix-assisted laser desorption/ionization (MALDI) of peptides with the hydrogen-donating matrix 5-amino-1-naphthol (5,1-ANL). MALDI-ISD performed at a low *M/A* of 6.3:1 resulted in unexpected backbone cleavages that formed (a + 16) and y-ions, while ISD spectra at *M/A* of 630:1 gave the usual fragments of c-, z'-, z-ANL and w-ions. Although the (a + 16) and y-ions observed at lower *M/A* values could not be rationalized by conventional ISD reactions, they were in agreement with the radiolytic products caused by the oxidation of peptides with hydroxyl radical HO•. MALDI mass spectra obtained at low *M/A* showed oxidized analyte ions $[M+nO]^+$ (n = 1–4). In addition, the spectra acquired in the low mass range showed peaks corresponding to $H^{\pm}$, $H_3O^{\pm}$, $H_2O^+$ (or $NH_4^+$), $HO^-$, $O_2^-$ and $HO_2^-$. These results suggest that MALDI-ISD experiments performed at lower *M/A* values can generate active radical species such as HO•, H• and $HO_2$•, which leads to the oxidative radical driven cleavage of peptides.



## 1. Introduction

Matrix-assisted laser desorption/ionization (MALDI) is a powerful method for elucidating the mass and structure of biomolecules such as peptides and proteins [1,2]. The mechanisms of MALDI with an ultraviolet (UV) laser have been reviewed [3–6]. Briefly, the principal ionization processes involve the formation of matrix radicals [M−H]•, hydrogen atom H•, matrix radical ions $M^{\bullet+}$ and $M^{\bullet-}$, and electrons $e^-$ via photochemical and photophysical events [7,8]. Electronic excitation of matrix occurs within fs ($10^{-15}$ s), and explosive ablation within ns to μs ($10^{-9}$–$10^{-6}$) after UV irradiation. Furthermore, depending on the physicochemical nature of matrix and analyte molecules, the ion-to-neutral ratios ejected into the gas-phase regions of the ion source are in the range of $10^{-7}$–$10^{-3}$ [9]. Hence, in MALDI, radical species are formed preferentially to ionic ones. The radical species such as hydrogen atoms generated under UV/MALDI conditions have provided basic and practical interests in peptide and protein sciences [10–13]. We have to notice unexpected reactions with radical species owing to high reactivity.

Hydrogen atoms H• and peptide radicals [M+H]• generated by MALDI in-source decay (ISD) play a very important role in the sequencing of peptides and top-down sequencing of proteins [10,11]. The peptide radicals [M+H]• immediately degrade at the N—Cα bond of the peptide backbone within ns in the ion source [12,13]. Use of the hydrogen-donating matrix 5-amino-1-naphthol (5,1-ANL) [14] in MALDI-ISD experiments has enabled estimation of flexible amino acid residues of proteins and phosphorylation site determination in proteins [15,16], as well as rapid sequencing of intact proteins [11]. MALDI-ISD with 5,1-ANL mainly results in the formation of c, z' and z-ANL ions that originate from radical-mediated fragmentation (RMF), as shown in Scheme 1 [15]. For MALDI-ISD experiments of peptides and proteins, it is important to set the conditions of laser fluence just above the ionization threshold (5–10% higher) and the range of matrix-to-analyte molar ratio (*M/A*) of 5000:1–10,000:1 [11,17].

In the course of MALDI-ISD experiment of peptides, we encountered the observation of unexpected fragment ions which may be originated from the inhomogeneity in *M/A* of sample crystal loaded on the MALDI plate. Here we attempt to employ lower *M/A* samples for MALDI-ISD experiment of peptides. It is shown with two different peptides that unexpected cleavages of peptide backbone can occur in MALDI-ISD experiments that are dependent on *M/A*. Use

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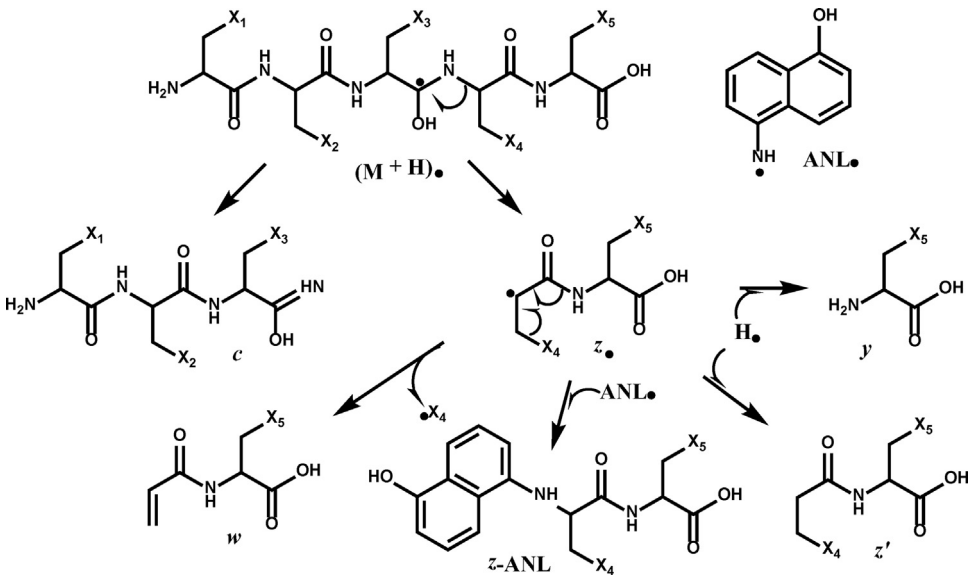


**Scheme 1.** Fragments generated from MALDI-ISD of peptides obtained with the hydrogen-donating matrix 5-amino-1-naphthol (5,1-ANL).

of the lower *M/A* ratios resulted in unusual fragments (a + 16) and y-ions that originated from the cleavage at Cα—CO and CO—NH bonds of the backbone, respectively. Although the generation of (a + 16) and y-ions could not be rationalized by the conventional ISD reactions in Scheme 1, they suggested that the oxidative radical driven cleavage at Cα—CO and CO—NH bonds of peptides occurred by the oxidation with hydroxyl radicals HO•.

## 2. Material and methods

### 2.1. Reagent and sample preparation

Peptide ACTH18-39 (RPVKVYPNGAEDESAEAFPLEF, *M*r 2465.7) and a synthetic peptide RAG12Leu (RAGFLAGTASALAALAALFL, *M*r 1905.3) were purchased from Peptide Institute (Minoh, Osaka, Japan). The MALDI matrix 5-amino-1-naphthol (5,1-ANL, *M*r 159) was bought from Tokyo Chemical Industry (Tokyo, Japan). Acetonitrile was 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.

For the MALDI-ISD experiments, the analyte was dissolved in water at a concentration of 100 pmol/μL. The matrix material was dissolved in water/acetonitrile (1:1, v/v) at a concentration of 63,000 pmol/μL without any additives. This matrix solution was diluted with water/acetonitrile (1:1, v/v) to concentrations of 6300, 630 and 63 pmol/μL. Samples were prepared by mixing 15 μL of analyte with 15 μL of matrix solution. The final *M/A* prepared for MALDI-ISD experiments was of 630:1, 63:1, 6.3:1 and 0.63:1. Sample solutions (0.5 μL) were deposited onto a stainless-steel MALDI plate and the solvents removed by evaporation in air at room temperature.

### 2.2. MALDI mass spectrometry

MALDI mass spectra were acquired on a time-of-flight mass spectrometer AXIMA-CFR (Shimadzu, Kyoto, Japan) equipped with a nitrogen laser (337 nm wavelength, 4 ns pulse width) operating at a pulse rate of 10 Hz. The laser spot size on the target substrate was ca. 100 μm in diameter. The laser fluence used for 5,1-ANL/peptide systems was changed in the range of 500–1300 J/m$^2$. The ions generated by MALDI were accelerated using 20 kV with delayed extraction. The analyzer was operated in positive- and negative-ion reflectron modes. The ions were detected using a microchannel plate detector. In total, 500 shots were accumulated for each mass spectrum.

## 3. Results and discussion

### 3.1. Unexpected preferential y-ion formation in MALDI-ISD of ACTH18-39

Positive- and negative-ion MALDI-ISD spectra of analyte peptide ACTH18-39 prepared at *M/A* of 630:1 are shown in Fig. 1. This *M/A* was within the normal range employed in the MALDI analysis of peptides and proteins. It was therefore expected that ISD reactions with 5,1-ANL would occur as shown in Scheme 1. Indeed, the positive-ion ISD spectrum mainly showed amino (N)-terminal side c-ion peaks. In addition, c6 and c18 ions were not detected due to the presence of Pro7 and Pro19 residues. By contrast, c-ions and carboxyl (C)-terminal side z'-, z-ANL, y- and w-ions were observed in negative-ion MALDI-ISD spectra. The ISD fragments c, z', z-ANL, y and w of peptides with 5,1-ANL are formed via prompt radical-mediated fragmentation (RMF) within ns ($10^{-9}$ s) in the MALDI ion source [12]. The detection of positive and negative fragment ions is mainly governed by the respective position of basic and acidic amino acid residues within a peptide. It must be noted, however, that MALDI-ISD occurs independently of ionization (protonation/deprotonation) [18]. The fragment ions observed in positive- and negative-ion spectra were assigned, as shown in the top panel of Fig. 1. The negative-ion spectrum showed prominent peaks corresponding to c11–c21 ions due to the presence of an acidic amino acid residue Glu11, although the c9 and c10 ions peaks were small (Fig. 1b). All the C-terminal z/y-ions from z7/y7 to z20/y20 and the corresponding z-ANL ions indicated by asterisks in Fig. 1b were observed except for z16 and z16-ANL. Detection of the latter ions was suppressed by Pro7 residue. The w-ions originated from the loss of radical X• from the side-chains (Scheme 1) and were characteristically observed at Asp, Asn and Glu residues.

In the course of negative-ion MALDI-ISD experiment of ACTH18-39, on the other hand, unexpected ISD spectral pattern was obtained when the perimeter of sample crystal was irradiated with UV laser, as shown in Fig. 2a and inset. The observed fragment ions were assigned as y- and (a + 16)-ions, while c-, z-, z-ANL and w-ions

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