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Selective Screening of Tyrosine-Nitrated Peptides in Tryptic Mixtures by In-Source Photodissociation at 355 nm in Matrix-Assisted Laser Desorption Ionization

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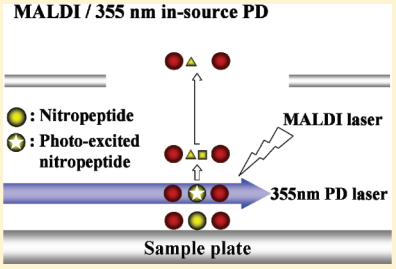
Supporting Information

ABSTRACT: Nitration of tyrosine residues in proteins is an important post-translational modification related to various diseases such as Alzheimer's. In this work, efficient and selective photodissociation (PD) at 355 nm was observed for $[M + H]^+$, $[M + H - 16]^+$, and $[M + H - 32]^+$ generated by matrix-assisted ultraviolet laser desorption ionization (UV-MALDI) of tyrosine-nitrated peptides (nitropeptides). Product ion spectra obtained by post-source PD at this wavelength contained useful information on the amino acid sequence. The spectra for nitropeptides obtained with 355 nm irradiation inside the ion source (MALDI/in-source PD) displayed characteristic triplet patterns due to PD of the above ions. For peptides displaying prominent signal in a MALDI mass map of a tryptic mixture, which are mostly those with arginine at the C-terminus, in-source PD allowed positive identification of their tyrosine-nitrated forms. Identification of such nitropeptides was possible at the 10 fmol level (in tryptic digest of 100 fmol BSA).



Nitration of tyrosine to 3-nitrotyrosine is one of the post-translational modifications of proteins that alters their activity.[1,2] Elevated levels of tyrosine-nitrated proteins were reported to be associated with various diseases, such as cardiovascular disease, Alzheimer's disease, arthritis, etc.

Mass spectrometry for peptides formed by tryptic digestion of a protein can be a sensitive technique to identify tyrosine-nitrated proteins and to locate the sites of the modification.[3−8] Mass spectral characteristics for peptides containing nitrotyrosine, to be called nitropeptides, have been widely investigated.[3,4,7,8] Matrix-assisted ultraviolet laser desorption ionization (UV-MALDI) of such peptides is known to generate not only intact peptide ions ($[M + H]^+$) but also fragment ions with one or two oxygen atom losses formed by photochemical degradation. In addition, peaks with m/z corresponding to $[M + H - 30]^+$ appear, which are due to photoinduced reduction of a nitro group to an amino group. Appearance of a quartet of peaks in the molecular ion region can be utilized as a signature for nitropeptides. However, sharing of peptide ion intensity by the quartet can be a handicap in the analysis of nitropeptides present at low levels in a tryptic mixture.[4] Potential problems in tandem mass spectrometry were noted as well,[6] such as the difficulty in precursor ion selection for post-source decay (PSD) and collisionally activated dissociation (CAD).

A widely used alternative is to convert 3-nitrotyrosine to 3-aminotyrosine and find peaks displaying m/z 30 shifts in the MALDI mass map.[6,9−11] Collection, and hence enrichment, of aminopeptides involved in this approach can be useful to identify nitropeptides present at low levels in a peptide mixture. In addition to amination, the technique also requires chemical derivatization before amination to protect the amino groups at the N-terminus and at the lysine side chain and derivatization of the amino group in 3-aminotyrosine after amination, which can be a potential handicap.

Unlike most of the activation techniques used in tandem mass spectrometry, photodissociation (PD) in the UV spectral range is specific to chromophores present in a peptide ion—other advantages of UV-PD include the absence of a need to introduce a collision gas, ease in monoisotopic selection of a precursor ion, and so forth.[12] In excitation with outputs from routinely available commercial lasers, side chains of aromatic residues such as phenylalanine, tryptophan, and tyrosine are the chromophores at 266 nm, while amide bonds are the chromophores at 193 and 157 nm.[12,13] For 3-nitrotyrosine in acidic solution, the absorption maximum is at 357−360 nm.[8] Hence, there is a possibility to selectively excite and dissociate gas-phase nitropeptide ions using the third harmonic (355 nm) of an Nd:YAG laser, which is widely available.

In this work, it will be shown that PD at 355 nm occurs efficiently for peptide ions containing 3-nitrotyrosine, while those without such a residue are unaffected. By 355 nm irradiation inside a MALDI source, it was possible to identify nitropeptides in a tryptic mixture, as will be demonstrated for those containing arginine at the C-terminus (C-arginine peptides).

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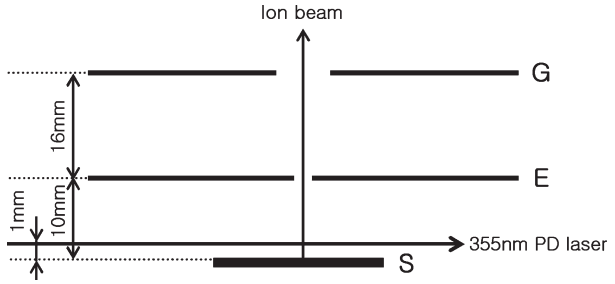

Figure 1. A schematic drawing of the ion source in the MALDI/in-source PD instrument used to obtain MALDI and in-source PD spectra. It consists of three electrodes S (sample plate), E, and G (ground). The distances between S and E and between E and G are 10 and 16 mm, respectively. The PD laser is cylindrically collimated (1.5 ×3 mm with 1.5 mm along the ion beam direction) and crosses the ion beam perpendicularly. The distance between the central axis of the laser beam and the sample plate is 1 mm.

EXPERIMENTAL SECTION

Instruments. Two MALDI-TOF instruments equipped with a reflectron, which will be referred to as MALDI-tandem TOF and MALDI/in-source PD, were used. The difference between the two is in the design of the ion source and reflectron. Details of MALDI-tandem TOF used to record post-source PD spectra for mass-selected ions were reported previously.[14,15] The potential inside its reflectron has both linear and quadratic components, which allows good product ion resolution without reflectron voltage stepping. The laser pulse for post-source PD perpendicularly crosses the first time focusing position of the instrument in synchronization with the lowest m/z isotopomer pulse of a precursor ion. The reflectron in the second instrument used to record in-source PD is a conventional design with a linear potential inside. A schematic drawing of the ion source in MALDI/in-source PD is shown in Figure 1. As in conventional design, the ion source consists of three electrodes S (sample plate), E, and G (ground). A difference is that the separation between S and E in this instrument is larger, 10 mm, for easy introduction of the PD laser without irradiating the sample. When optimized, larger electrode separation leads to longer delay time and hence longer reaction time for photoexcited peptide ions. The PD laser is cylindrically collimated (1.5 ×3 mm, 1.5 mm along the ion beam direction) and crosses the ion beam perpendicularly. The distance between the laser beam and the sample plate is 1 mm. Taking the time of MALDI laser irradiation as time zero, the PD laser is fired at t = 1.47 μs. Initially, both S and E are kept at 20.0 kV. Then, the voltage on S is raised to 21.9 kV at t = 2.14 μs for delayed extraction. A total of 25.0 kV is applied to the final electrode of the reflectron. A nitrogen laser (MNL205-C, Lasertechnik, Berlin, Germany) is used for MALDI. The third harmonic of an Nd:YAG laser (355 nm, Surelite III-10, Continuum, Santa Clara, CA, USA) is used for in- and post-source PD.

Data Acquisition and Treatment. The method to acquire and treat post-source PD data was reported previously.[14] Briefly, a precursor ion beam is roughly selected by the ion gate and photoexcited by synchronization of the ion beam pulse with the PD laser pulse, which allows monoisotopic selection. A laser-off tandem mass spectrum (PSD) is subtracted from a laser-on spectrum to obtain a PD spectrum. The method to obtain an in-source PD spectrum is similar, except that mass selection cannot be achieved.

The method to treat MALDI spectra for a tryptic mixture obtained without and with in-source PD laser irradiation, i.e., MALDI and MALDI/in-source PD mass maps, is as follows. The extent of photodepletion is calculated for peaks with intensities larger than a threshold level. Peaks displaying photodepletion larger than 50% are selected as the candidates for nitropeptides (50% PD criterion). Among these, peaks forming a triplet with each pair separated by m/z 16 or a doublet separated by m/z 16 or 32 were chosen (triplet/doublet criterion, to be explained).

Samples. Tryptic digest of bovine serum albumin (BSA) was purchased from Waters (Milford, MA, USA). YLYEIAR, HPEYAVSVLLR, NYQEAK, IGDYAGIK, IGSEVYHNLK, and their nitrated counterparts were purchased from Peptron (Daejeon, Korea). The first three peptides are part of BSA, while the fourth and fifth are in alcohol dehydrogenase and enolase, respectively. The matrix α-cyano-4-hydroxycinnamic acid (CHCA) and other chemicals were purchased from Sigma (St. Louis, MO, USA).

Matrix solution was prepared by dissolving 10 mg of CHCA in 1 mL of 1:1 mixture of acetonitrile and trifluoroacetic acid (TFA). Peptide aqueous solution and matrix solution were mixed and loaded on the sample plate. Around 100 pmol of each peptide was loaded in each spot in the study of pure peptides, while an amount in femtomole range was used in the analytical study of tryptic mixtures.

RESULTS AND DISCUSSION

Post-source PD Spectra. As mentioned earlier, MALDI of nitropeptides (to be shown later) generated prominent peaks at m/z corresponding to $[M + H]^+$, $[M + H - 16]^+$, $[M + H - 30]^+$, and $[M + H - 32]^+$. Due to the limited resolution of the ion gate in the MALDI-tandem TOF instrument, it was difficult to record a contamination-free PSD spectrum for each ion. In contrast, post-source PD spectra could be obtained for monoisotopic precursor ions through ion beam-laser beam synchronization. $[M + H]^+$, $[M + H - 16]^+$, and $[M + H - 32]^+$ underwent efficient PD at 355 nm. PD efficiencies for the first two ions were comparable, while that of the third was a little lower. The ion containing an aminotyrosine residue, i.e., the peak corresponding to $[M + H - 30]^+$, hardly underwent PD even when laser intensity was raised substantially. Post-source PD spectra for $[M + H]^+$, $[M + H - 16]^+$, and $[M + H - 32]^+$ from $_n$YLYEIAR—$_n$Y denotes nitrotyrosine residue—obtained with 0.8 mJ/pulse at 355 nm are shown in Figure 2. Around 20% of precursor ions underwent PD at this pulse energy.

As is characteristic of UV-PD for peptide ions containing arginine,[12] product ions formed by charge-directed cleavage, i.e., a_2, b_n (n = 2, 3), y_n (n = 1−6), y_n-NH_3 (n = 1−4), and those formed by charge-remote cleavage, i.e., v_n (n = 3, 5), w_n (n = 4−6), and w_{3a}, appear in the PD spectrum of $[_n$YLYEIAR + H$]^+$. In addition, immonium ions, i.e., I (and/or L), E, R, and $_n$Y, several internal acyl ions such as YE, and nitrotyrosine side chain loss peak (MH-152) are present. Appearance of complete y series together with w_{3a} and w_6 allows definite sequencing for the peptide. One thing to note is that the b_4 ion, which is the largest peak in the PSD spectrum (not shown), registers a negative signal in the PD spectrum. This occurs when a PSD product ion moving together with the precursor ion undergoes PD. Also to be noted is the absence of $[M + H - 16]^+$ and $[M + H - 32]^+$ peaks. This suggests that the corresponding ions in the MALDI spectrum are not formed by gas-phase dissociation of $[M + H]^+$. The nitro group remained intact also in PSD and CAD spectra

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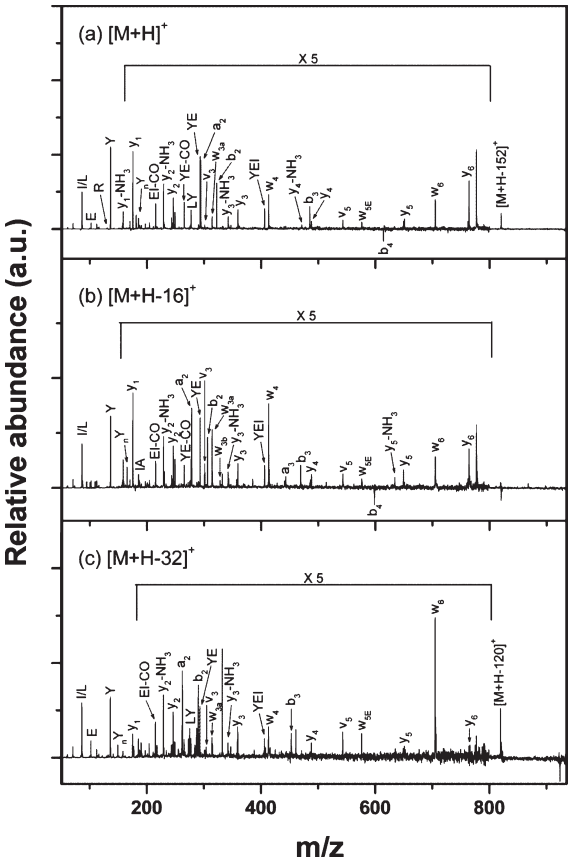

Figure 2. Post-source PD spectra for (a) $[M + H]^+$ (m/z 972.5), (b) $[M + H - 16]^+$ (956.5), and (c) $[M + H - 32]^+$ (940.5) from $_n$YLYEIAR obtained with 0.8 mJ/pulse at 355 nm. $_n$Y in (a) denotes nitrotyrosine immonium ion while those in (b) and (c) denote the corresponding ions with one and two oxygen losses, respectively. The m/z values of the same a_n or b_n ions in the three spectra are also different because of mass difference in nitrotyrosine parts. Around 20% of monoisotopic precursor ions were depleted by PD. Laser-off spectrum was subtracted from laser-on spectrum.

reported previously.[3,4,7] Product ions appearing in PD spectra for $[M + H - 16]^+$ and $[M + H - 32]^+$ are remarkably similar to those for $[M + H]^+$. For example, sequence ions such as a_n (n = 2, 3), b_n (n = 2, 3), y_n (n = 1−6), v_n (n = 3, 5), w_n (n = 4−6), w_{3a}, and w_{3b} are prominent product ions from $[M + H - 16]^+$. The m/z values of the same a_n or b_n ions in the three spectra are different by 16 or 32 because of the mass difference in nitrotyrosine parts in the three precursor ions.

MALDI/In-Source PD. MALDI spectrum of $_n$YLYEIAR is shown in Figure 3(a). As mentioned earlier, $[M + H]^+$ is accompanied by $[M + H - 16]^+$ and $[M + H - 32]^+$. The isotopic pattern of $[M + H - 32]^+$ looks unusual due to the presence of $[M + H - 30]^+$. Other prominent peaks are mostly matrix (CHCA)-related, as marked in the figure. Product ions formed by in-source decay (ISD) of the peptide ion also appear, even though weakly. They are very similar to those formed by post-source PD; sequence ions a_2, b_n (n = 2−4), y_n (n = 1−6), y_n-NH_3 (n = 1−3, 5, 6), v_n (n = 3, 5), w_n (n = 4, 6), and w_{3a}, immonium ions I (and/or L), E, and $_n$Y, internal acyl ions such as YE, and MH-152. ISD product ions are very weak except for the immonium ions and MH-152.

In in-source PD at 355 nm, a sufficiently large laser energy (10 mJ/pulse) was used to deplete more than 50% of the peptide ion intensity. The in-source PD spectrum, i.e., laser-off subtracted from laser-on, for $_n$YLYEIAR is shown in Figure 3(b). In this spectrum, negative- and positive-going signals are due to photodepletion and photoformation, respectively. Neglecting the photodepletion of matrix-related peaks, appearance of a triplet of very strong negative signals for $[M + H]^+$, $[M + H - 16]^+$, and $[M + H - 32]^+$ is the outstanding feature of this spectrum. Unlike post-source PD, in-source PD mostly generates product ions with low m/z such as immonium ions due to multiphoton absorption at high laser intensity. Among the product ions, MH-152 displays the largest negative signal, which is a potential source of false-positives when one attempts to identify nitropeptides in a tryptic mixture based on in-source PD. More worrisome are the corresponding ions formed from un-nitrated peptides with tyrosine. Unless the analysis of nitropeptides present at very low level is attempted, this would not be critical because MH-152 is much weaker than $[M + H]^+$ in MALDI spectra. Also, false-positives originating from MH-152 can be eliminated because the extent of PD for this ion is much less than that for $[M + H]^+$ and because it does not display triplet or doublet patterns in in-source PD.

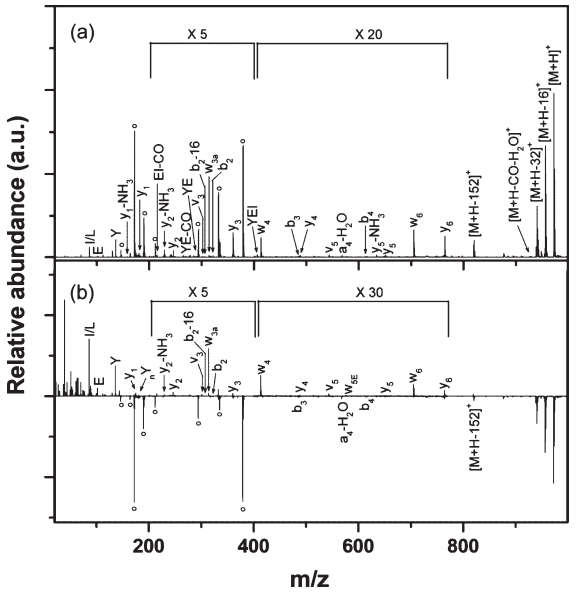

Figure 3. (a) MALDI and (b) in-source 355 nm PD spectra of $_n$YLYEIAR. Ten mJ/pulse of PD laser depleted 55% of the peptide ion signal. Fragment ions in (a) are those generated by in-source decay (ISD) in MALDI. Matrix-related peaks are marked with circles (○).

In-Source PD of Nitropeptides Added to BSA Tryptic Mixture. Even though tyrosine residues in BSA are not nitrated in its native form, some of them can be converted to nitrotyrosine by protein nitration.[16] In fact, mass spectrometric detection of $_n$YLYEIAR[3,4] formed by nitration of BSA has been a subject of interest. As an attempt to evaluate the capability of 355 nm in-source PD in identifying nitropeptides, we studied the tryptic digest of BSA injected with C-arginine nitropeptides. The first thing we noted was that when a C-arginine peptide appeared prominently in MALDI mass map, its nitrated form also did. In that case, the nitrated form, when present in sufficient amount, could be readily identified from its efficient photodepletion at 355 nm.

To demonstrate the utility of in-source PD under the proteomics condition, MALDI spectra recorded without and with in-source 355 nm irradiation obtained from tryptic digest of

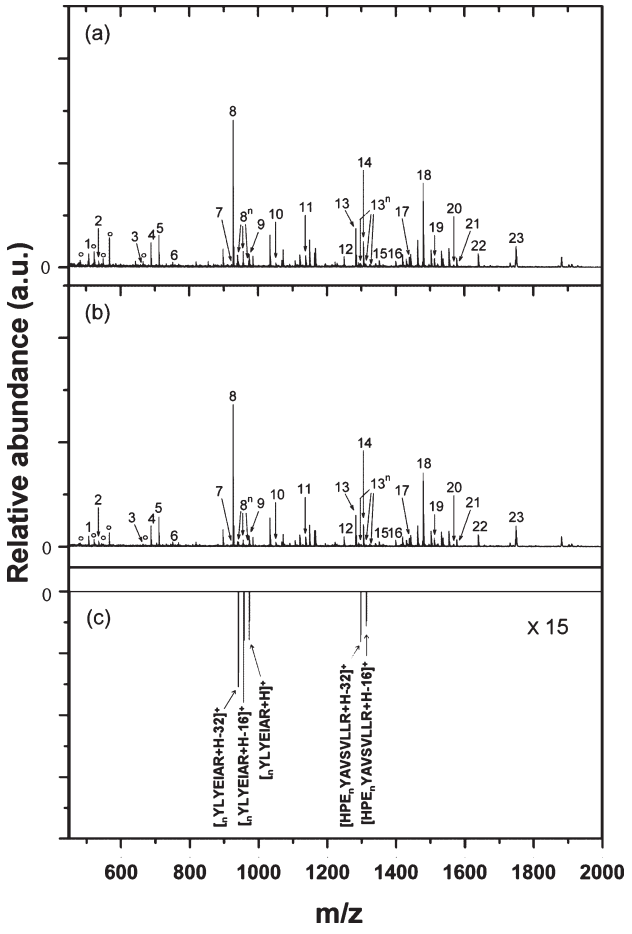

Figure 4. (a) Laser-off and (b) 355 nm laser-on MALDI spectra obtained from a spot containing tryptic digest of 100 fmol BSA spiked with 10 fmol of $_n$YLYEIAR and 20 fmol of HPE$_n$YAVSVLLR. Identity of each peptide ion peak can be found in Table 1. Nitrated forms of the peptides 8 and 13 are marked as 8^n and 13^n. Matrix-related peaks are also marked (○). Each spectrum was obtained by averaging over 1000 MALDI shots. (c) 355 nm in-source PD spectrum obtained by subtracting (a) from (b) and imposing the 50% PD and triplet/doublet criteria, multiplied by 15.

100 fmol BSA spiked with 10 fmol of $_n$YLYEIAR and 20 fmol of HPE$_n$YAVSVLLR are shown in Figure 4(a). Each spectrum was obtained by averaging over 1000 MALDI shots at one sample spot. Peptides in the MALDI mass map are numbered in the figure (up to 2000 Da) and listed in Table 1. Among 60 tryptic peptides that can be formed in BSA tryptic digestion, 15 can be identified. In addition, 8 peptides formed by miss cleavage appear. Prominent peaks are mostly due to C-arginine peptides. Peak #8 due to YLYEIAR is the most prominent, while #13 due to HPEYAVSVLLR is not as prominent. In addition, a triplet of peaks from $_n$YLYEIAR is easily recognized, while that from HPE$_n$YAVSVLLR is not. When the MALDI mass map was searched for triplets of peaks, with each pair separated by m/z 16, these two peptides were found. However, quite a few false-positives also appeared in such a search. That is, the MALDI mass map alone was not useful for positive identification of nitropeptides.

In-source irradiation of 355 nm PD laser hardly changed the MALDI spectrum (laser-on) as shown in Figure 4(b), indicating that the peptide ions formed by MALDI of BSA tryptic mixture did not undergo PD at this wavelength. Closer inspection shows

Table 1. Peptides (below 2000 Da) Appearing in MALDI Mass Map of BSA Tryptic Digest

ID	peptide	MH^+	ID	peptide	MH^+
1	FGER	508.3	13	HPEYAVSVLLR	1283.7
2	FWGK	537.3	14	HLVDEPQNLIK	1305.7
3	KFWGK	665.4	15	VTKCCTESLVNR	1352.7
4	AWSVAR	689.4	16	TVMENFVAFVDK	1399.7
5	SEIAHR	712.4	17	RHPEYAVSVLLR	1439.8
6	NYQEAK	752.4	18	LGEYGFQNALIVR	1479.8
7	AEFVEVTK	922.5	19	VPQVSTPTLVEVSR	1511.8
8	YLYEIAR	927.5	20	DAFLGSFLYEYSR	1567.7
9	DLGEEHFK	974.5	21	ECCHGDLLECADDR	1578.6
10	CCTKPESER	1052.4	22	KVPQVSTPTLVEVSR	1639.9
11	CASIQKFGER	1138.6	23	LSQKFPKAEFVEVTK	1751.0
12	FKDLGEEHFK	1249.6			

that the intensities of $_n$YLYEIAR triplet and those of some matrix-related ions decreased upon laser irradiation. In contrast, photodepletion of HPE$_n$YAVSVLLR triplet is difficult to recognize. As described in the Experimental Section, we subtracted the laser-off MALDI spectrum (Figure 4(a)) from the laser-on spectrum (Figure 4(b)) and applied the 50% PD and triplet/doublet criteria to find nitropeptides. The result is shown in Figure 4(c). Even though some matrix-related peaks underwent efficient PD at 355 nm, they did not appear in Figure 4(c) due to the triplet/doublet criterion. The remaining peaks are solely due to $_n$YLYEIAR and HPE$_n$YAVSVLLR. In the case of HPE$_n$YAVSVLLR, $[M + H]^+$ did not pass the 50% PD criterion because of the presence of another ion at the same m/z that did not undergo PD and hence is absent in Figure 4(c). Frequent overlapping of nondissociating ions with one of the peaks in a molecular ion triplet was the reason why the second criterion was loosened to include doublets. In the case of HPE$_n$YAVSVLLR, the more massive of the doublet was identified as $[M + H - 16]^+$ based on the presence of a distinct peak at m/z higher by 16 and the absence of such a peak at m/z lower by 32 in the MALDI spectrum.

Other Peptides. Even though the present technique is very simple to use, it is not applicable to peptides that appear weak or do not appear at all in MALDI mass map. This is often the case for C-lysine peptides. Since they are weaker bases than C-arginine peptides, they might lose in competition for protons to the latter and hence do not appear as ionic species.[17] Conversion of lysine to homoarginine by guanidination, which increases the basicity of a C-lysine peptide, has been a popular technique to improve the peptide coverage in MALDI mass map.[18] We investigated 355 nm PD of C-lysine nitropeptides after conversion of their lysine residues to homoarginine by guanidination. Overall mass spectral features for such peptides were very similar to C-arginine nitropeptides. $[M + H]^+$, $[M + H - 16]^+$, $[M + H - 30]^+$, and $[M + H - 32]^+$ were formed by MALDI. $[M + H - 30]^+$ did not undergo PD at 355 nm while the other three did. In the post-source 355 nm PD spectrum for guanidinated N$_n$YQEAK—shown in the Supporting Information—a_2, b_n (n = 2, 3), y_n (n = 1−4), y_n-NH_3 (n = 1−4), v_5, and w_n (n = 3, 4) were prominent features, just as for $_n$YLYEIAR. We also injected several C-lysine peptides and their nitrated counterparts to BSA tryptic digest, guanidinated the mixture, and investigated its MALDI and in-source PD at 355 nm. We found that the abundance of $[M + H]^+$ of a C-lysine nitropeptide in MALDI

spectrum of the mixture almost paralleled that of its un-nitrated counterpart. That is, the former ion was prominent when the latter ion was prominent and was absent when the latter was absent. Positive identification by in-source PD at 355 nm was possible for C-lysine nitropeptide ions appearing prominently in the mixture spectrum—shown in the Supporting Information. However, somewhat larger amounts were needed than those in the above direct analysis, probably due to sample loss in the guanidination step.

We also investigated 355 nm PD, both in- and post-source, of various peptide ions without and with other post-translational modifications (PTM). Ordinary peptide ions without PTM did not display noticeable PD at this wavelength regardless of their compositions—absorption and fluorescence in near UV and visible are known for some proteins.[19,20] One would expect to encounter a 355 nm chromophore when an aromatic residue in a peptide is modified by PTM just as in the case of 3-nitrotyrosine. In this regard, we attempted but failed to induce PD at 355 nm for tyrosine-phosphorylated peptides. Among other PTMs, nitrosylation of cysteine is known to produce peptides and proteins absorbing at 340 nm. However, no negative-going signal was observed in in-source PD of S-nitrosylated peptides at 355 nm. This was probably because their molecular ions dissociated rapidly after MALDI as reported previously.[21] Nitrosation of proteins and nitration and oxidation of tryptophan may generate chromophores at 355 nm.[22] Utility of MALDI/in-source PD at 355 nm for the identification of such proteins needs to be further checked.

CONCLUSIONS

We have shown that post-source PD at 355 nm is a useful and selective technique for identification of tryptic nitropeptides. However, this tandem mass spectrometric technique may not be quite useful for identifying small amounts of nitropeptides present in tryptic mixtures. MALDI/in-source PD at 355 nm investigated in this work, which does not require preseparation of peptide ions and measures precursor ion signals rather than product ion signals, can be advantageous in this regard. Even though the present report has been focused on the positive identification of nitropeptides in tryptic mixtures, the technique can also identify true-negatives, i.e., peptides without nitrotyrosine, which can be useful to find tyrosine residues unaffected by protein nitration.

The main problem of the present technique lies in the limited peptide coverage in MALDI of peptide mixtures. Even though the conversion of lysine to homoarginine improved the peptide coverage, sample loss in the guanidination step led to deterioration in detection sensitivity. Use of other ionization techniques, such as electrospray ionization, and use of other proteolytic enzymes, such as chymotrypsin, may help to extend the peptide coverage.

ASSOCIATED CONTENT

Supporting Information. (1) Post-source 355 nm PD spectrum of a guanidinated C-lysine nitropeptide. (2) Identification of C-lysine nitropeptides injected to BSA tryptic digest by in-source 355 nm PD after guanidination. (3) List of peptides appearing in MALDI mass map of BSA tryptic digest after guanidination. This material is available free of charge via the Internet at http://pubs.acs.org.

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