Efficient Identification and Quantification of Peptides Containing Nitrotyrosine by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry after Derivatization

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Protein nitration at tyrosine residues proceeds to form 3-nitrotyrosine; this product is today used as a biomarker of nitrative stress. We have developed an efficient method with which to identify nitrated peptides and to quantify protein nitration levels in different biological samples by a combination of the chemical derivatization of nitrotyrosine-containing peptides and mass spectrometry. Our strategy includes: 1) the protection of both N-terminal amines and ε-amines of lysine residues by acetylation with $^{13}C_0/^{13}C_4$– or D_0/D_6–acetic anhydride; 2) the reduction of nitrotyrosine to aminotyrosine with sodium hydrosulfite; and 3) the derivatization of aminotyrosine with 1-(6-methyl[D_0/D_3]nicotinoyloxy)succinimide. The utility of our method is demonstrated with nitrotyrosine-containing angiotensin II and bovine serum albumin as the model compounds.

Key words nitrotyrosine; derivatization; quantification; matrix-assisted laser desorption/ionization time-of-flight MS

Protein nitration at tyrosine residues leading to 3-nitrotyrosine (NO_2–Tyr) is one of the nonenzymatic post-translational modifications that can occur in cells under conditions of nitrosative stress.[1] There are two main pathways of protein nitration. One is the generation of peroxynitrite ($ONOO^-$) by the reaction of nitric oxide ($^{\cdot}NO$) with superoxide ($O_2^{\cdot-}$) and the subsequent reaction of $ONOO^-$ with CO_2, which leads to the generation of nitrogen dioxide ($^{\cdot}NO_2$) and the carbonate radical ($CO_3^{\cdot-}$).[2] The other is the generation of $^{\cdot}NO_2$ by the reaction of nitrite (NO_2^-) with hydrogen peroxide (H_2O_2) in the presence of heme proteins, such as myeloperoxidase.[3] Tyrosine nitration can alter protein structure and function by forming NO_2–Tyr, partly because of the shift in the pK_a value of the tyrosine hydroxyl group from 10.1 to 7.2.[4] Tyrosine nitration may also prevent tyrosine phosphorylation, which is important in signal transduction.[5] Increased levels of protein nitration have been observed in various diseases,[6—12] such as cardiovascular disease,[9] Alzheimer's disease,[10,12] and Parkinson's disease.[11] Because protein nitration is associated with both physiological and pathological conditions, it is necessary to develop efficient methods to identify the nitration sites and quantify the nitration levels in proteins.

NO_2–Tyr is a sufficiently stable modification to be used as a biomarker of $^{\cdot}NO/ONOO^-$-related damage. Several methods have been developed to detect protein nitration.[12—16] Immunodetection with antibodies directed against nitrotyrosine is one of the most commonly used methods.[12] Many high-performance liquid chromatography (HPLC)-based methods with ultraviolet,[13] electrochemical,[14] and fluorescent detection[15] have also been reported. However, these methods are based on the detection and/or quantification of free nitrotyrosine, either in plasma or generated after the cleavage of the peptide bond by enzymatic or acidic hydrolysis.[16] These methods yield quantitative data only regarding the changes in nitration levels in whole tissues or whole proteins, and do not identify the sites of NO_2–Tyr in proteins.

Recently, mass spectrometry (MS)-based methods have been developed as an important technique for the identification of nitrated proteins and nitrated sites in proteins. Mass spectrometers with matrix-assisted laser desorption/ionization (MALDI)[17—19] or electrospray ionization (ESI)[20—25] have been widely used in these studies. The application of tandem mass spectrometry (MS/MS) makes it possible to identify nitrated sites in proteins.[20—25] In the MALDI analysis of nitrotyrosine-containing peptides, the characteristic mass pattern derived from nitrotyrosine has mass values lower than that of $[M+H]^+$ by 16 Da and 32 Da. These ions correspond to the loss of one and two oxygen atoms, respectively, from the nitro group ($-NO_2$), and are generated during the ionization process with laser irradiation. Because the signals are split, the signal intensity of $[M+H]^+$ is reduced. However, these mass patterns are not observed in ESI analysis.

Other approaches include MS analysis coupled to the chemical derivatization of nitrotyrosine for its enrichment[26,27] and specific detection.[28,29] These methods involve the reduction of nitrotyrosine to aminotyrosine, followed by its specific chemical derivatization. The derivatization reaction is carried out at pH 5.0, exploiting the lower basicity of aminotyrosine (pK_a=4.7). The N-terminal amino groups and ε-amino groups of lysine residues have higher pK_a values of *ca.* 8 and *ca.* 9.5, respectively, and are largely protonated at pH 5.0. For example, Nikov *et al.* attached a cleavable biotin tag to aminotyrosine at pH 5.0 to purify the biotinylated tryptic peptides using a streptavidin affinity column.[26] Zhang *et al.* reported an enrichment strategy with improved derivatization specificity and high-efficiency capture of nitrotyrosine peptides based upon a highly specific cysteinyl peptide enrichment technique.[27] Amoresano *et al.* reported the selective modification of the nitration sites with dansyl chloride and the selective detection and identification of the modified peptides using the characteristic fragmentation of the dansyl derivative.[28] More recently, Chiappetta *et al.* reported a strategy based on the use of isobaric tag for relative and absolute quantification (iTRAQ) reagents coupled to LC-MS/MS for quantitative identification of nitration sites in protein and demonstrated the utility of the strategy in model proteins and

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in more complex sample.[29] By using a precursor ion scanning (PIS) for the iTRAQ reporter ions, chromatogram of nitrated bovine serum albumin (BSA) recorded in PIS was clearly simplified in comparison to chromatogram of nitrated BSA recorded in full scan. In the case of nitrated BSA, four nitration sites were identified and quantified.

In this study, we report a novel strategy to expand the utility of MS analysis of nitrotyrosine-containing peptides derived from protein digestion, which can identify the nitrated peptides and quantify the nitration levels of proteins in different biological samples by the specific derivatization of nitrotyrosine. Our labeling strategy includes: 1) the protection of the N-terminal amines and ε-amines of lysine residues by acetylation with acetic anhydride; 2) the reduction of nitrotyrosine to aminotyrosine with sodium hydrosulfite; and 3) the derivatization of aminotyrosine with 1-(6-methylnicotinoyloxy)succinimide.[30,31] To demonstrate the use of our method, nitrated angiotensin II (Ang II) and BSA are used as model compounds.

Experimental

Materials Ang II (DRVYIHPF, $[M+H]^+=1046.54$) was purchased from the Peptide Institute, Inc. (Osaka, Japan). Nitrated angiotensin II (NO_2–Ang II, DRVY(NO_2)IHPF, $[M+H]^+=1091.53$) was custom-made by Sigma-Aldrich Japan (Tokyo, Japan). BSA, α-cyano-4-hydroxycinnamic acid (CHCA), ammonium bicarbonate (NH_4HCO_3), sodium hydrosulfite ($Na_2S_2O_4$), and trypsin (proteomics grade) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), triethylammonium bicarbonate buffer (TEAB) was from Fluka (St. Louis, MO, U.S.A.), and acetic anhydride (Ac_2O), HPLC-grade acetonitrile (CH_3CN), and trifluoroacetic acid (TFA) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetic anhydride (D_6, 98%) and acetic anhydride ($^{13}C_4$, 99%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.). 1-(6-Methylnicotinoyloxy)succinimide (**1**) and 1-(6-methyl[D_3]nicotinoyloxy)succinimide (**2**) were prepared in-house, as reported previously.[30,31]

Derivatization of Model Peptides Acetylation: Ang II and nitrated Ang II (NO_2–Ang II) were dissolved in water at a concentration of 1 mM. Equal amounts of the two peptide solutions were mixed to produce a 1 mM peptide solution containing Ang II and NO_2–Ang II (1 : 1). After 100 mM tetraethylammoniumbromide (TEAB) (50 μl) and Ac_2O (10 μl) had been added to the peptide mixture (50 μl), the reaction mixture was incubated at 37 °C for 30 min. Then, 18% ammonia solution (100 μl) was added and incubated at 37 °C for another 30 min. The reaction mixture was finally concentrated by evaporation.

Reduction and Derivatization: The acetylated peptides were dissolved in 100 mM TEAB (50 μl). Next, 50 mM $Na_2S_2O_4$ in 100 mM TEAB (50 μl) was added and the reaction mixture was stirred at room temperature for 30 min to reduce 3-nitrotyrosine to 3-aminotyrosine. To the reduced peptide solution was added 200 mM amine-reactive reagent in 50% CH_3CN (25 μl) and the reaction mixture was stirred at room temperature for another 30 min. Then, 18% ammonia solution (25 μl) was added and the reaction mixture was stirred at room temperature for a further 1 h. The reaction was quenched by the addition of 50% TFA (20 μl) and the mixture was diluted with water (80 μl) to produce a 0.2 mM peptide solution. An aliquot of the derivatized solution (10 μl) was diluted with water (90 μl) and the resulting solution (0.5 μl, 10 pmol) was subjected to MALDI-time of flight (TOF) MS analysis.

Nitration and Tryptic Digestion of BSA BSA was dissolved in 100 mM NH_4HCO_3 at a concentration of 2 μg/ml. Peroxynitrite solution was prepared using a stock solution stored at −80 °C. The concentrations of $ONOO^-$ were determined by spectrophotometry ($\varepsilon=1670\,M^{-1}cm^{-1}$, $\lambda=$ 301 nm in 0.1 M NaOH). Trypsin was dissolved in 1 mM HCl at a concentration of 1 μg/μl. The BSA solution (200 μl) was nitrated by the addition of 0—30 mM peroxynitrite solution (10 μl). The reaction mixture was stirred with a vortex mixer for 10 s and allowed to stand at room temperature for 1 h. Next, 100 mM NH_4HCO_3 (190 μl) was added, producing a 1 μg/ml solution, which was stored at −30 °C before use. Trypsin solution (2 μl) was added to the stock solution of $ONOO^-$-treated protein solution (100 μl) and the reaction mixture was incubated at 37 °C for a further 15 h.

Derivatization of Tryptic Peptides Acetylation: The tryptic peptides (10 μl, 10 μg) were mixed with 100 mM TEAB (10 μl). After Ac_2O (2 μl) was added, the reaction mixture was incubated at 37 °C for 30 min. Then, 18% ammonia solution (20 μl) was added and the reaction mixture was incubated at 37 °C for another 30 min. The reaction mixture was finally concentrated by evaporation.

Reduction and Derivatization: The acetylated peptides were dissolved in 100 mM TEAB (20 μl). Then, 50 mM $Na_2S_2O_4$ in 100 mM TEAB (10 μl) was added and the reaction mixture was stirred at room temperature for 30 min to reduce 3-nitrotyrosine to 3-aminotyrosine. To the reduced peptide solution was added 200 mM amine-reactive reagent in 50% CH_3CN (10 μl) and the reaction mixture was stirred at room temperature for another 30 min. Then, 18% ammonia solution (10 μl) was added and the reaction mixture was stirred at room temperature for a further 1 h. The reaction was quenched by the addition of 50% TFA (10 μl) and the mixture was diluted with water (140 μl) to produce a 50 ng/μl peptide solution. An aliquot of the derivatized solution (10 μl) was desalted and concentrated to 5 μl (100 ng/μl) with ZipTip$_{C18}$ (Millipore, Bedford, MA, U.S.A.). An aliquot of the resulting solution (0.5 μl, 50 ng) was subjected to MALDI analysis.

MALDI-TOF MS Analysis MALDI-TOF mass spectra were acquired on an AXIMA-CFR instrument (Shimadzu, Kyoto, Japan). The operating conditions were as follows: nitrogen laser, 337 nm; reflectron mode; positive ion detection; and acceleration voltage, 20 kV. Each spectrum was obtained by accumulating 50 shots. High-purity CHCA (proteomics grade) was used as the MALDI matrix throughout this study, without further purification. CHCA was dissolved in 50% CH_3CN in 0.05% TFA at a concentration of 10 mg/ml. The samples were prepared for MS analysis by mixing 0.5 μl of the reaction mixture with 0.5 μl of the matrix solution on the MALDI target plate. The applied samples were allowed to dry at room temperature.

Database Search Peptide mass fingerprint (PMF) search was carried out using MASCOT search engine (Matrix Science Inc., Boston, MA, U.S.A.) with the National Center for Biotechnology Information database (NCBI, Bethesda, MD, U.S.A.). The search parameters were as follows: Taxonomy, metazoan (animals); enzyme, trypsin; fixed modifications, none; variable modifications, none; mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±0.2 Da; peptide charge state, 1+; and max missed cleavages, 1.

Sample Preparation and ESI-MS Analysis The derivatized peptides were desalted with MonoTip® C18 (GL Sciences Inc., Tokyo, Japan) and subjected to liquid chromatography (LC)-MS/MS. ESI-MS analysis was performed with an ESI ion trap mass spectrometer (LCQ, Thermo Electron Corporation, San Jose, CA, U.S.A.) equipped with a nanospray ion source. The extracted peptides were loaded onto a trap cartridge similar to a C8 solid phase (Peptide Micro Trap, AMR Inc., Osaka, Japan), followed by a Unison C18 reversed-phase nano-LC column (0.1×150 mm; Imtakt, Kyoto, Japan), and then directly subjected to ESI-MS analysis. HPLC separations were performed on an HPLC system (Shimadzu, Japan) using gradient elution with solvent A (5% CH_3CN with 0.05% formic acid) and solvent B (90% CH_3CN with 0.05% formic acid), at a flow rate of 0.055 ml/min. The elution conditions consisted of two segments with increasing concentrations of solvent B: 10—100% gradient. The mass scanning range of the instrument was set at m/z 300—2000. The ion spray voltage was set at 2.5 kV in the positive-ion mode. Data-dependent MS/MS spectra were acquired by automatic switching between the MS and MS/MS modes (with the collision energy set at 35%) using dynamic exclusion, which temporarily puts a mass onto an exclusion list after its mass spectrum is acquired to avoid obtaining repetitive data for abundant peptides. Helium was used as the collision gas.

Results and Discussion

Our strategy to identify and quantify the NO_2–Tyr-containing peptides involves the chemical derivatization of the peptide in three steps, as shown in Fig. 1. In the first step, both the N-terminal amine and the ε-amine of the lysine in the peptide are blocked by acetylation with acetic anhydride (Ac_2O) or isotope-labeled Ac_2O (D_6 or $^{13}C_4$). In the second step, NO_2–Tyr is converted to 3-aminotyrosine (NH_2–Tyr) by reduction with sodium hydrosulfite ($Na_2S_2O_4$).[26—29] In the third step, the amino group of NH_2–Tyr is derivatized with an amine-reactive reagent (**1** or **2**) at pH 8.4. In our strategy, all the peptides were acetylated in the first step, with a mass increase of 42 Da. In contrast, the reduction and derivatization reactions are specific to NO_2–Tyr-containing peptides, with

characteristic mass shifts of −30 Da and +119 Da, respectively. This method is useful for the efficient identification, using the characteristic mass shifts, of the NO_2–Tyr-containing peptides in a peptide mixture derived from a protein digestion. Moreover, because unmodified and nitrated peptides are chemically labeled in the acetylation step and at both the acetylation and derivatization steps, the changes in the unmodified/nitrated ratio can be easily estimated. In this study, Ang II and BSA were used as the model compounds to demonstrate the utility of our method.

Derivatization of Model Peptides Containing NO_2–Tyr

To demonstrate our derivatization method, equal amounts of a mixture of Ang II ($[M+H]^+=1046.54$) and NO_2–Ang II ($[M+H]^+=1091.53$) were used as the model peptides.[19,20] A mixture of Ang II and NO_2–Ang II (1 : 1) was acetylated with Ac_2O, reduced with $Na_2S_2O_4$, and derivatized with amine-reactive reagent **1**. At the acetylation and derivatization steps, the hydroxy group (–OH) of the tyrosine residue was also derivatized. To remove this unwanted side reaction, the reaction mixture was incubated with the addition of ammonia solution at each step. The MALDI-TOF mass spectra are shown in Fig. 2. Figure 2a shows the MALDI-TOF mass spectrum of the underivatized peptide mixture containing equal amounts of Ang II and NO_2–Ang II. In the MALDI analysis, the NO_2–Tyr-containing peptide displayed the characteristic mass pattern, as shown in Fig. 2a. Thus, the two signals were observed at *m*/*z* 1075.60 and 1059.61, which are lower than the signal for NO_2–Ang II at *m*/*z* 1091.58 by 16 Da and 32 Da, respectively. This is because one and two oxygens have been lost, respectively, from the nitro group (–NO_2), as described in a previous report.[18—20,28] Similarly, as shown in Fig. 2b, the characteristic mass pattern was observed in which the mass number is shifted +42 Da from that shown in Fig. 2a after acetylation with Ac_2O. Conversely, the signal corresponding to the NH_2–Tyr-containing-peptide was observed at *m*/*z* 1103.59 after the reduction of the nitro group to an amino group (–NH_2) with $Na_2S_2O_4$ (Fig. 2c). Thus, the reduction of the nitro group with the characteristic mass shift (−30 Da) is useful for the identification of NO_2–Tyr-containing-peptides. However, the signal intensity of the NH_2–Tyr-containing-peptide at *m*/*z* 1103.59 was lower than that of acetylated Ang II (Ac–Ang II) at *m*/*z* 1088.60, even when

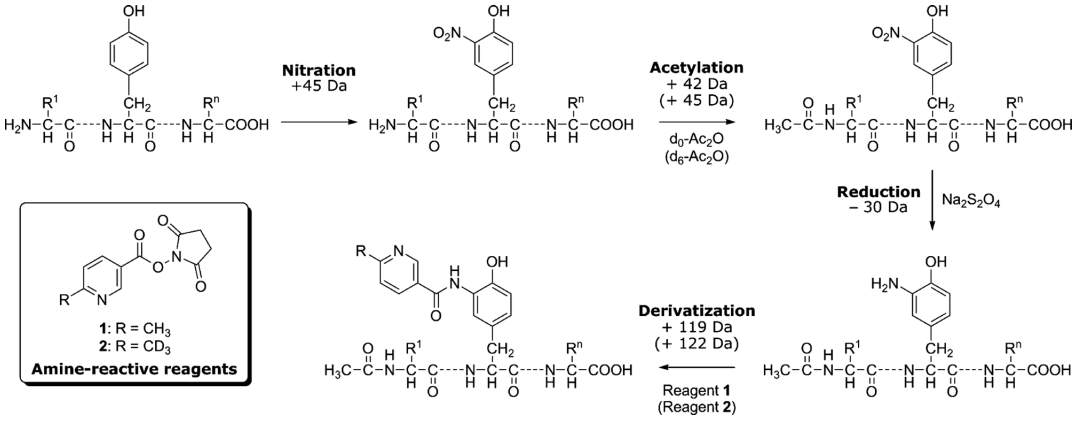


Fig. 1. Strategy for the Specific Derivatization of Nitrotyrosine-Containing Peptides, and the Chemical Structures of Amine-Reactive Reagents **1** and **2**

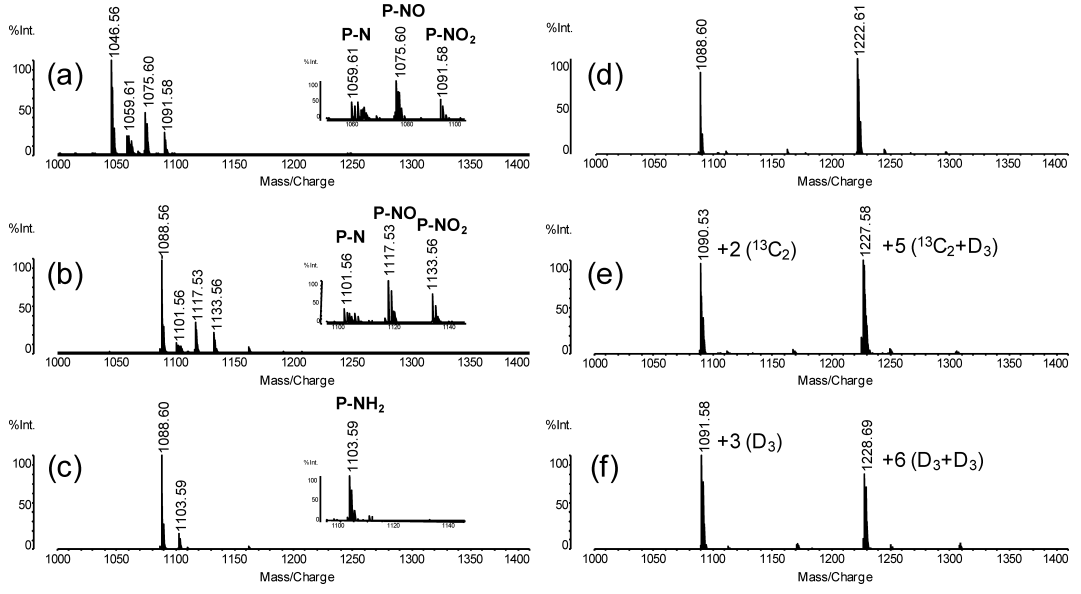


Fig. 2. MALDI-TOF Mass Spectra of a Mixture of Equal Amounts of Angiotensin II (DRVYIHPF, $[M+H]^+=1046.54$) and Nitrated Angiotensin II (DRVY(NO_2)IHPF, $[M+H]^+=1091.53$)

(a) Before derivatization. (b) After acetylation with Ac_2O. (c) After reduction with $Na_2S_2O_4$. (d) After derivatization with reagent **1**. (e) As for (d), except for acetylation with $^{13}C_4$–Ac_2O and derivatization with reagent **2**. (f) As for (d), except for acetylation with D_6–Ac_2O and derivatization with reagent **2**.

equal amounts of them were subjected to MALDI-TOF MS analysis. In this study, the resulting NH_2–Tyr was derivatized with amine-reactive reagent **1** to enhance the ionization efficiency. After derivatization with reagent **1**, no signal for the NH_2–Tyr-containing-peptide was observed at *m*/*z* 1103.59, whereas the signal of the peptide derivatized with reagent **1** was observed at *m*/*z* 1222.61 (+119 Da) with almost the same signal intensity as that of Ac–Ang II observed at *m*/*z* 1088.60 (Fig. 2d). Thus, this derivatization was useful for the identification of the nitrated peptide with the enhanced ionization efficiency in MALDI analysis. Probably, the enhancement of signal intensity is due to the excitation of peptide derivatized with reagent **1** by UV photons from nitrogen laser. Unfortunately, the addition of reagent **1** only brings the signal intensity of NH_2–Tyr-containing-peptide to the same level as the Ac–Ang II. The signal intensity depends on the sequence of the peptide. In the case of Arg-containing peptides such as Ang-II, the signal enhancement by derivatization was not so remarkable.[31] Figures 2e and f show the MALDI-TOF mass spectra of the same peptide mixture derivatized with $^{13}C_4$–Ac_2O/reagent **2** (D_3) and D_6–Ac_2O/reagent **2** (D_3), respectively, instead of with Ac_2O/reagent **1**. The signal for Ac–Ang II observed at *m*/*z* 1088.60 (Fig. 2d) was observed at *m*/*z* 1090.53 (Fig. 2e) and 1091.58 (Fig. 2f), with mass increases of 2 Da and 3 Da, respectively. In contrast, the signal of the peptide derivatized with Ac_2O/reagent **1** observed at *m*/*z* 1222.61 (Fig. 2d) was observed at *m*/*z* 1227.58 (Fig. 2e) and 1228.69 (Fig. 2f), with mass increases of 5 Da and 6 Da, respectively. Thus, with our chemical derivatization method, both the native and nitrated peptides were derivatized with isotope-labeled reagents. Therefore, we think that our method could be useful in calculating the degree of nitration from the decrease of native peptide using MALDI-TOF mass spectrometry.

MS Analysis of Nitrated Peptides from Tryptic Peptides of BSA BSA was treated with 10 mM $ONOO^-$ and digested with trypsin. MALDI mass spectra of these tryptic peptides were taken and analyzed. We identified 18 different tryptic peptides of BSA that corresponding to 28% of sequence coverage. The identified tryptic peptides were summarized in Table 1. A spectrum of nitrated peptide was shown in Fig. 3b, which was manually searched in comparison to the spectrum of untreated control (Fig. 3a). In Fig. 3b, the characteristic mass patterns of the NO_2–Tyr-containing peptide were observed at *m*/*z* 940.46 (P-N), 956.43 (P-NO), and 972.44 (P-NO_2) with mass losses of 16 Da, respectively, as already demonstrated for NO_2–Ang II (Fig. 2a). These peaks were corresponded to those derived from the peptide YLYEIAR of which one Tyr residue was nitrated (*m*/*z* 972.44). A signal at *m*/*z* 927.45 in Fig. 3b indicates that the nitration proceeded partially.

The tryptic peptides of $ONOO^-$-treated BSA were acetylated with Ac_2O, reduced with $Na_2S_2O_4$, and derivatized with amine-reactive reagent **1**. The resulting peptide mixture was desalted and subjected to MALDI-TOF MS analysis. The partial MALDI-TOF mass spectra are shown in Fig. 4. Figure 4a shows the MALDI-TOF mass spectrum of tryptic BSA treated with $ONOO^-$. Figures 4b and c show the MALDI-TOF mass spectra of the nitrated peptides after acetylation with Ac_2O and reduction with $Na_2S_2O_4$, respectively. Accompanying the disappearance of the characteristic mass pattern,

Table 1. Experimental Data of Tryptic Peptides of BSA

No.	Start—end	Observed	Sequence	Number of Tyr (position)
1	223—228	649.24	CASIQK	0
2	156—160	665.28	KFWGK	0
3	236—241	689.31	AWSVAR	0
4	29—34	712.31	SEIAHR	0
5	161—167	927.45	YLYEIAR	2 (161, 163)
6	37—44	974.42	DLGEEHFK	0
7	588—597	1050.43	EACFAVEGPK	0
8	66—75	1163.58	LVNELTEFAK	0
9	35—44	1249.61	FKDLGEEHFK	0
10	361—371	1283.70	HPEYAVSVLLR	1 (364)
11	402—412	1305.70	HLVDEPQNLIK	0
12	360—371	1439.80	RHPEYAVSVLLR	1 (364)
13	421—433	1479.79	LGEYGFQNALIVR	1 (424)
14	347—359	1567.75	DAFLGSFLYEYSR	2 (355, 357)
15	437—451	1639.95	KVPQVSTPTLVEVSR	0
16	469—482	1667.84	MPCTEDYLSLILNR	1 (475)
17	508—523	1823.90	RPCFSALTPDETYVPK	1 (520)
18	168—183	2044.96	RHPYFYAPELLYYANK	4 (171, 173, 179, 180)

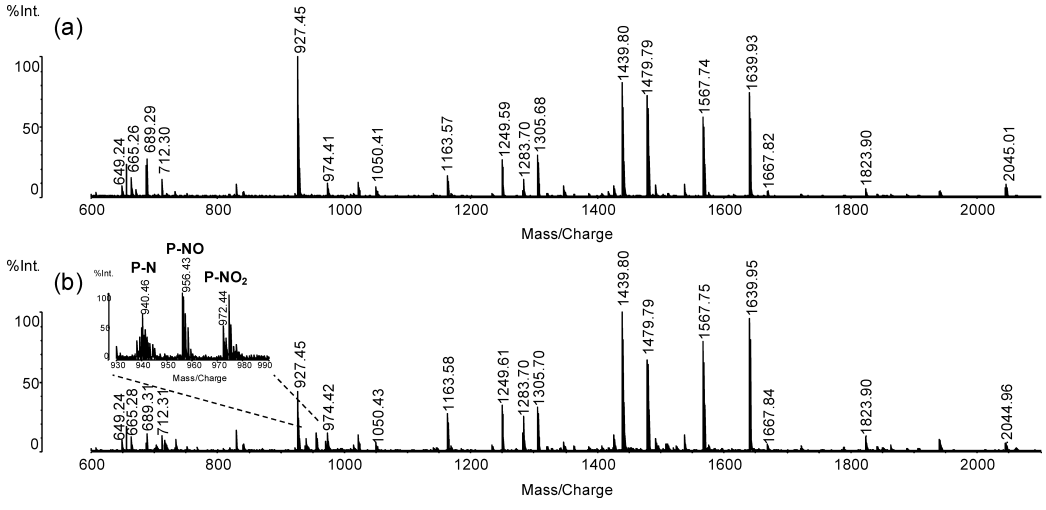


Fig. 3. MALDI-TOF Mass Spectra of Tryptic Peptides of BSA Untreated (a) and Treated (b) with $ONOO^-$

a new signal corresponding to the NH_2–Tyr-containing peptide (P-NH_2) was observed at *m*/*z* 984.51 (Fig. 4c). After derivatization with reagent **1**, the signal for the peptide was observed at *m*/*z* 1103.59, with a mass shift of +119 Da and the signal intensity was enhanced relative to the signal intensity at *m*/*z* 969.51 (Fig. 4d). However, the peptide YLYEIAR contains two Tyr residues in the sequence. To confirm the nitration site, the derivatized peptide was analyzed with LC-MS/MS.

MS/MS Analysis of Derivatized Nitrotyrosine-Containing Peptide Figure 5a shows the MS/MS spectrum of the $[M+H]^+$ ion (*m*/*z* 1103) corresponding to the peptide YLYEIAR acetylated with Ac_2O and derivatized with reagent **1**. A series of b-ions (b_1—b_6) shows that the N-terminal Tyr residue (Tyr161) is the nitrated site. In contrast, Fig. 5b shows the MS/MS spectrum of the $[M+H]^+$ ion (*m*/*z* 1108), corresponding to the peptide YLYEIAR acetylated with $^{13}C_4$–Ac_2O and derivatized with amine-reactive reagent **2** (D_3). In this spectrum, all the b-ions (b_1—b_6) are shifted by 5 Da towards the higher mass range. These results confirm that the N-terminal Tyr161 is nitrated because both the b_1 and b_2 ions are observed at the same mass values if Tyr163 is nitrated. The basicity of the pyridine moiety is smaller than that of the side chain of Arg residue. However, the signal intensity of b-ions having pyridine moiety were stronger than that of y-ions having Arg residue as shown in Fig. 5. The result is consistent with previous work by Münchbach *et al.*[32]

We identified 18 different tryptic peptides of BSA (Fig. 3a, Table 1). Among them, 8 peptides (No. 5, 10, 12—14, 16—18 in Table 1) contain 12 Tyr residues (Tyr161, 163, 171, 173, 179, 180, 355, 357, 364, 424, 475, 520). Under our conditions, nitration of Tyr161 in peptide YLYEIAR was only found, and the nitration site was consistent with the one previously reported for BSA nitration.[18,19,25,28]

Quantitative Analysis of BSA Nitration To examine the utility of our method in quantitative analyses, BSA was used as the model protein and treated with different concentrations of $ONOO^-$ solution. One sample (sample A) was treated with $ONOO^-$ solution (0, 5, 10, 20, or 30 mM) and digested with trypsin. The tryptic peptides were acetylated with Ac_2O, reduced with $Na_2S_2O_4$, and derivatized with reagent **1**. The other sample (sample B) was treated with $ONOO^-$ solution (10 mM) and digested with trypsin. The tryptic peptides were acetylated with D_6–Ac_2O, reduced with $Na_2S_2O_4$, and derivatized with reagent **2** (D_3). Equal amounts of samples A and B were mixed and subjected to MALDI-TOF MS analysis. Quantitative analysis was carried out by using the isotope-coded peptides (sample B) as internal stan-

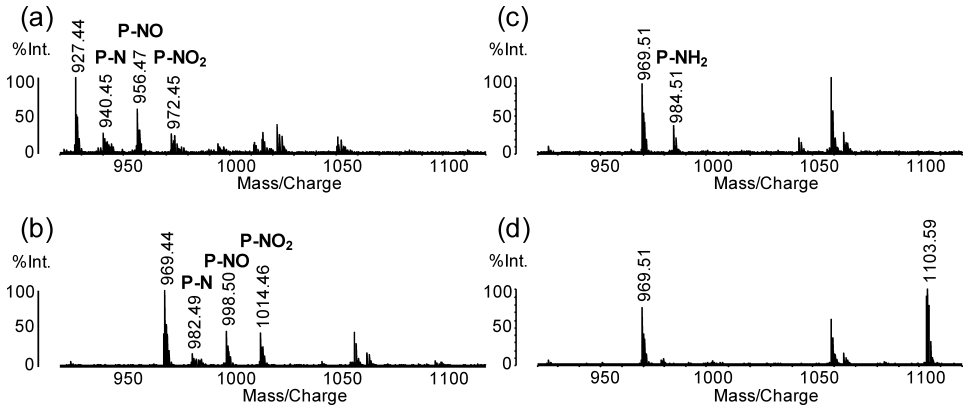


Fig. 4. Partial MALDI-TOF Mass Spectra of Tryptic Peptides of BSA Treated with $ONOO^-$

(a) Before derivatization. (b) After acetylation with Ac_2O. (c) After reduction with $Na_2S_2O_4$. (d) After derivatization with reagent **1**.

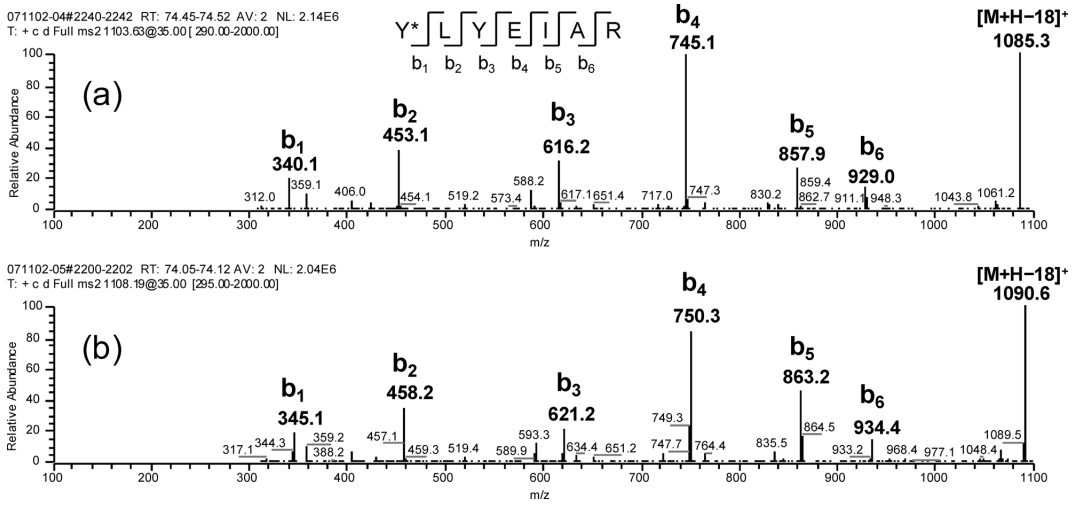
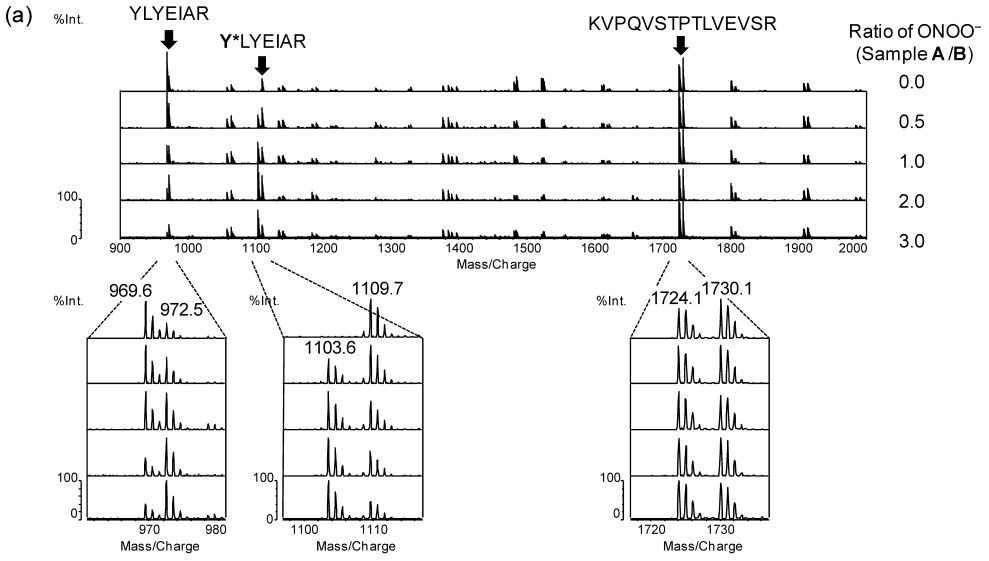


Fig. 5. MS/MS Spectra of the Nitrated Peptide within the BSA Sequence

(a) After acetylation with Ac_2O and derivatization with reagent **1**. (b) After acetylation with $^{13}C_4$–Ac_2O and derivatization with reagent **2**.



(b)

Ratio of $ONOO^-$ (Sample **A**/**B**)	Ratio of Signal Intensity (Sample **A**/**B**)			Degree of nitration (Decrease of native peptide)
	m/z 969.6/972.5	*m/z* 1103.6/1109.7	*m/z* 1724.1/1730.1	
0.0	2.53 ± 0.32	0.00 ± 0.00	0.83 ± 0.03	0 %
0.5	1.55 ± 0.12	0.67 ± 0.01	0.98 ± 0.07	39 %
1.0	1.01 ± 0.04	1.33 ± 0.09	1.13 ± 0.03	60 %
2.0	0.49 ± 0.03	1.58 ± 0.04	0.98 ± 0.05	81 %
3.0	0.38 ± 0.02	2.22 ± 0.14	1.11 ± 0.08	85 %

Fig. 6. Quantitative Analysis of the Nitration of BSA

Sample A was treated with different concentrations of $ONOO^-$ solution (0, 5, 10, 20, 30 mM). Sample B was treated with 10 mM $ONOO^-$ solution. After tryptic digestion, samples A and B were derivatized with Ac_2O/reagent **1** and D_6–Ac_2O/reagent **2**, respectively. A mixture of equal amounts of the derivatized samples A and B was subjected to MALDI-TOF MS analysis. The ratios of the concentration of $ONOO^-$ treatment in the mixture of samples A and B were A/B=0.0, 0.5, 1.0, 2.0, and 3.0. (a) MALDI-TOF mass spectra. (b) Summary of ratio of signal intensity.

dards.

In this study, the native peptides were observed as doublet peaks spaced by 3 Da (D_0/D_3). On the other hand, the nitrated peptides were observed as doublet peaks spaced by 6 Da (D_0+D_0/D_3+D_3). As shown in Fig. 6a, the native peptides (YLYEIAR) and the nitrated peptides (Y*LYEIAR) were observed at *m/z* 969.6/972.5 and 1103.6/1109.7, respectively, as doublet peaks spaced by 3 Da and 6 Da, respectively. The ratios of signal intensity (A/B) were summarized in Fig. 6b. With increases in the $ONOO^-$ ratio (A/B=0.0, 0.5, 1.0, 2.0, 3.0), the signal intensity of *m/z* 969.6 decreased compared with that of *m/z* 972.5. The ratio of signal intensity (*m/z* 969.6/972.5) decreased from 2.53 to 0.38 (Fig. 6b). Conversely, the signal intensity of *m/z* 1103.6 increased relative to that of *m/z* 1109.7. The ratio of signal intensity (*m/z* 1103.6/1109.7) increased from 0.00 to 2.22 (Fig. 6b). These results show that the nitration levels of the peptide Y161 increased with increasing $ONOO^-$ concentrations. In peptides containing no Tyr residues, such as KVPQVSTPTLVEVSR (*m/z* 1724.1, 1730.1), the ratio of the signal intensities (*m/z* 1724.1/1730.1) was almost constant value, even when the concentration of $ONOO^-$ was increased. Moreover, the constant value of the peptide having no modification site shows that the total protein quantity is the same between the two samples A and B.

In the case of $ONOO^-$ ratio A/B=0.0, the peptide YLYEIAR in sample A is not nitrated. So, the degree of nitration could be calculated by the decrease of signal intensity at *m/z* 969.6 using the signal intensity at *m/z* 972.5 as internal standard. The ratios of signal intensity (*m/z* 969.6/972.5) 1.55, 1.01, 0.49, and 0.38 were decreased by 39%, 60%, 81%, and 85%, respectively, from 2.53. These degrees of signal decrease are corresponding to the degree of nitration.

Conclusions

We have described an efficient method for the identification and quantification of nitrotyrosine-containing peptides. Our strategy involves: 1) the protection of both the N-terminal amines and ε-amines of the lysine residues by acetylation with Ac_2O; 2) the reduction of nitrotyrosine to aminotyrosine with $Na_2S_2O_4$; and 3) the derivatization of the resulting aminotyrosine with amine-reactive reagents. The ionization efficiency of the derivatized peptide was clearly enhanced relative to that of aminotyrosine-containing peptides. This derivatization was useful for the identification of the nitrated site by MS/MS analysis with both the enhanced ionization efficiency of the fragment ions and the mass shift induced by the isotope-labeled reagents. The mass differences generated by **1** and **2** were used in the relative quantification of the protein nitration in two different samples using MALDI-TOF mass spectrometry. Our method should enable the analysis of the degree of protein nitration.

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References

1) Radi R., *Proc. Natl. Acad. Sci. U.S.A.*, **101**, 4003—4008 (2004).
2) Tien M., Berlett B. S., Levine R. L., Chock P. B., Stadtman E. R., *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 7809—7814 (1999).

3) Vliet A., Eiserich J.-P., Halliwell B., Cross C.-E., *J. Biol. Chem.*, **272**, 7617—7625 (1997).
4) Sokolovsky M., Riordan J.-F., Vallee B.-L., *Biochem. Biophys. Res. Commun.*, **27**, 20—25 (1967).
5) Newman D.-K., Hoffman S., Kotamraju S., Zhao T., Wakim B., Kalyanaraman B., Newman P.-J., *Biochem. Biophys. Res. Commun.*, **296**, 1171—1179 (2002).
6) Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A., *Mass Spectrom. Rev.*, **24**, 55—99 (2005).
7) Szabó C., Ischiropoulos H., Radi R., *Nat. Rev.*, **6**, 662—680 (2007).
8) Reynolds M.-R., Berry R.-W., Binder L.-I., *Biochemistry*, **46**, 7325—7336 (2007).
9) Peluffo G., Radi R., *Cardiovasc. Res.*, **75**, 291—302 (2007).
10) Castegna A., Thongboonkerd V., Klein J.-B., Lynn B., Markesbery W.-R., Butterfield D.-A., *J. Neurochem.*, **85**, 1394—1401 (2003).
11) Zhang L., Dawson V.-L, Dawson T.-M., *Pharmacol. Ther.*, **109**, 33—41 (2006).
12) Smith M.-A., Richey H.-P., Sayre L.-M., Beckman J.-S., Perry G., *J. Neurosci.*, **17**, 2653—2657 (1997).
13) Evans P., Kaur H., Mitchinson M.-J., Halliwell B., *Biochem. Biophys. Res. Commun.*, **226**, 346—351 (1996).
14) Hensley K., Maidt M.-L., Yu Z., Sang H., Markesbery W.-R., Floyd R.-A., *J. Neurosci.*, **18**, 8126—8132 (1998).
15) Kamisaki Y., Wada K., Ataka M., Yamada Y., Nakamoto K., Ashida K., Kishimoto Y., *Biochem. Biophys. Acta*, **1362**, 24—28 (1997).
16) Ryberg H., Caidahl K., *J. Chromatogr. B*, **851**, 160—171 (2007).
17) Sultana R., Reed T., Perluigi M., Coccia R., Pierce W.-M., Butterfield D.-A., *J. Cell. Mol. Med.*, **11**, 839—851 (2007).
18) Sarver A., Scheffler N.-K., Shetlar M.-D., Gibson B.-W., *J. Am. Soc. Mass Spectrom.*, **12**, 439—448 (2001).
19) Petersson A.-S., Steen H., Kalume D.-E., Caidahl K., Roepstorff P., *J. Mass Spectrom.*, **36**, 616—625 (2001).
20) Lee S.-J., Lee J.-R., Kim Y.-H., Park Y.-S., Park S.-I., Park H.-S., Kim K.-P., *Rapid Commun. Mass Spectrom.*, **21**, 2797—2804 (2007).
21) Kanski J., Hong S.-J., Schöneich C., *J. Biol. Chem.*, **280**, 24261—24266 (2005).
22) Sacksteder C.-A., Qian W.-J., Knyushko T.-V., Wang H., Chin M.-H., Lacan G., Melega W.-P., Camp D.-G., Smith R.-D., Smith D.-J., Squier T.-C., Bigelow D.-J., *Biochemistry*, **45**, 8009—8022 (2006).
23) Gokulrangan G., Zaidi A., Michaelis M.-L., Schöneich C., *J. Neurochem.*, **100**, 1494—1504 (2007).
24) Jiao K., Mandapati S., Skipper P.-L., Tannenbaum S.-R., Wishnok J.-S., *Anal. Biochem.*, **293**, 43—52 (2001).
25) Willard B.-B., Ruse C.-I., Keightley J.-A., Bond M., Kinter M., *Anal. Chem.*, **75**, 2370—2376 (2003).
26) Nikov G., Bhat V., Wishnok J.-S., Tannenbaum S.-R., *Anal. Biochem.*, **320**, 214—222 (2003).
27) Zhang Q., Qian W.-J., Knyushko T.-V., Clauss T.-R.-W., Purvine S.-O., Moore R.-J., Sacksteder C.-A., Chin M.-H., Smith D.-J., Camp II D.-G., Bigelow D.-J., Smith R.-D., *J. Proteome Res.*, **6**, 2257—2268 (2007).
28) Amoresano A., Chiappetta G., Pucci P., D'Ischia M., Marino G., *Anal. Chem.*, **79**, 2109—2117 (2007).
29) Chiappetta G., Corbo C., Palmese A., Marino G., Amoresano A., *Proteomics*, **9**, 1524—1537 (2009).
30) Tsumoto H., Murata C., Miyata N., Taguchi R., Kohda K., *Chem. Pharm. Bull.*, **51**, 1399—1401 (2003).
31) Tsumoto H., Murata C., Miyata N., Kohda K., Taguchi R., *Rapid Commun. Mass Spectrom.*, **21**, 3815—3824 (2007).
32) Münchbach M., Quadroni M., Miotto G., James P., *Anal. Chem.*, **72**, 4047—4057 (2000).