research articles Journal of proteome research

A Method for Selective Enrichment and Analysis of Nitrotyrosine-Containing Peptides in Complex Proteome Samples

Qibin Zhang,[¶,‡] Wei-Jun Qian,[¶,‡] Tatyana V. Knyushko,[†] Therese R. W. Clauss,[¶] Samuel O. Purvine,[¶] Ronald J. Moore,[¶] Colette A. Sacksteder,[†] Mark H. Chin,[#,§] Desmond J. Smith,[§] David G. Camp II,[¶] Diana J. Bigelow,[†] and Richard D. Smith*[,¶]

Biological Sciences Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99352, Cell Biology and Biochemistry Group, Pacific Northwest National Laboratory, Richland, Washington 99352, Department of Human Genetics, and Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA, Los Angeles, California 90095

Received December 21, 2006

Elevated levels of protein tyrosine nitration have been found in various neurodegenerative diseases and age-related pathologies. Until recently, however, the lack of an efficient enrichment method has prevented the analysis of this important low-level protein modification. We have developed a method that specifically enriches nitrotyrosine-containing peptides so that both nitrotyrosine peptides and specific nitration sites can be unambiguously identified with LC−MS/MS. The procedure consists of the derivatization of nitrotyrosine into free sulfhydryl groups followed by high efficiency enrichment of sulfhydryl-containing peptides with thiopropyl sepharose beads. The derivatization process includes: (1) acetylation with acetic anhydride to block all primary amines, (2) reduction of nitrotyrosine to aminotyrosine, (3) derivatization of aminotyrosine with *N*-Succinimidyl *S*-Acetylthioacetate (SATA), and (4) deprotection of *S*-acetyl on SATA to form free sulfhydryl groups. The high specificity of this method is demonstrated by the contrasting percentage of nitrotyrosine-derivatized peptides in the identified tandem mass spectra between enriched and unenriched samples. Global analysis of unenriched *in vitro* nitrated human histone H1.2, bovine serum albumin (BSA), and mouse brain homogenate samples had 9%, 9%, and 5.9% of identified nitrotyrosine-containing peptides, while the enriched samples had 91% , 62%, and 35%, respectively. Duplicate LC−MS/MS analyses of the enriched mouse brain homogenate identified 150 unique nitrated peptides covering 102 proteins with an estimated 3.3% false discovery rate.

Keywords: Nitrotyrosine peptide enrichment • nitrotyrosine derivatization • tyrosine nitration • sulfhydryl peptide enrichment • Histone 1.2 • BSA nitration, mouse brain • LC−MS/MS

Introduction

Tyrosine nitration of proteins is generated by reactive nitrogen species either from the inflammatory cell defense mechanism or from a combination of nitric oxide synthase activity with a high rate of oxidative metabolism.[1−5] Increased levels of nitrotyrosine in a variety of tissues have been identified in over 80 different pathologies,[6] including various neurodegenerative diseases[6−9] as well as in normal biological aging.[10−15] Tyrosine nitration can alter protein structure and function, affect its biological half-life, inactivate enzymes and receptors that depend on tyrosine residues for their activity, and prevent phosphorylation of tyrosine residues important for signal transduction.[15,16] In addition, recent work has shown that tyrosine nitration can have a regulatory role in redox signaling. In this regard, reversible nitration in response to redox conditions has been demonstrated in mitochondria for proteins critical for energy and antioxidant homeostasis.[17]

Nitrotyrosine has been suggested and used as a biomarker for diagnosis of oxidative stress-induced pathological conditions.[18−20] Typically, identification and quantification of nitrotyrosine has involved measuring the free nitrotyrosine either in plasma or generated by protein hydrolysis applying various detection methods, for example, LC−UV, LC−MS, LC−ECD, and GC−MS.[19−22] However, these methods only provide an overall level of nitration as no specific protein identification or nitration site information can be obtained.

* To whom correspondence should be addressed. Dr. Richard D. Smith, Biological Sciences Division, Pacific Northwest National Laboratory, P.O. Box 999, MSIN: K8-98, Richland, WA 99352. Fax: (509) 376-7722. E-mail: rds@pnl.gov.

¶ Biological Sciences Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory.

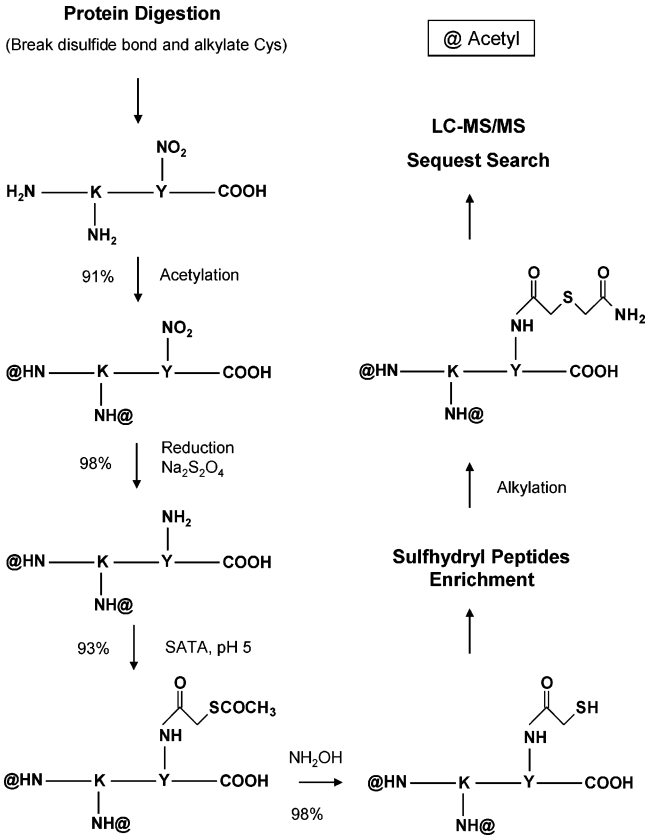
‡ These authors contributed equally.

† Cell Biology and Biochemistry Group, Pacific Northwest National Laboratory.

Department of Human Genetics, David Geffen School of Medicine at UCLA.

§ Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at UCLA.

Scheme 1. Steps Involved in the Enrichment and Analysis of Nitrotyrosine-Containing Peptides from a Protein Mixture[a]



[a] To illustrate the reactions involved, only the terminus, lysine and tyrosine of a peptide are displayed. The efficiency of each chemical reaction on a nitrotyrosine-containing standard peptide is shown, which was calculated based on plotting the selected ion chromatograms and considering all the charge states of each species. The detailed reaction conditions are shown in Experimental Procedures.

To correlate tyrosine nitration with any biological dysfunction and elucidate the role of nitrotyrosine in the signal transduction process, knowing the precise location and extent of tyrosine nitration within a given protein is necessary. To this end, various protein separation methods coupled with mass spectrometry (MS)-based bottom-up proteomic approaches have been applied to identify nitrotyrosine proteins and locate nitration sites.[15] Generally in these studies, different protein separation methods such as 2D-gel electrophoresis,[23,24] C_4 reversed-phase LC (RPLC),[25] and in-solution isoelectrofocusing[14] coupled with SDS-PAGE were used to separate the protein mixture followed by immunostaining with an anti-nitrotyrosine antibody. The nitrotyrosine-containing bands were identified by in-gel digestion and tandem MS (i.e., MS/MS) analysis. Although peptide mass fingerprinting has been used in earlier studies,[26] MS/MS has been proven to be the detection method of choice for unambiguous identification of tyrosine nitration sites.[15] New sites of tyrosine nitration have been identified from various tissues using the methods previously mentioned; however, typically only a few nitrotyrosine proteins have been identified due to limitations that are associated with analysis sensitivity, nitrotyrosine antibody specificity, and protein separation methods.[27−29] More recently, we have applied multidimensional LC (SCX coupled with RPLC)−MS/MS to extensively characterize the mouse brain proteome, which resulted in the identification of 31 unique nitrotyrosine sites in 29 different proteins.[6] More than half of the nitrated proteins identified were involved in neurodegenerative diseases.[6] Although this approach shows advantages over previously reported gel-based methods, the detection of many lower abundant nitrotyrosine proteins remains problematic.

For proteome-wide analysis of this low-abundance protein modification, a specific enrichment approach prior to MS analysis is highly desirable. To date, no effective nitrotyrosine peptide enrichment approach has been reported. One study recently reported an enrichment strategy involving the reduction of 3-nitrotyrosine to 3-aminotyrosine, coupling to a cleavable biotin affinity tag, and capturing with a streptavidin affinity column.[30] This approach was sound in principle, but suffered from the limitations of nonspecific derivatization reactions to aminotyrosine in the presence of other primary amines as well as of the nonspecific binding during avidin affinity purification. Indeed, this approach has only been demonstrated for single proteins. Here, we report a novel enrichment strategy with improved derivatization specificity and high efficiency capture of nitrotyrosine peptides based upon a highly specific cysteinyl (or sulfhydryl-containing) peptide enrichment strategy previously developed in our laboratory.[31] The effectiveness of this approach coupled with MS for nitrotyrosine site identification is evaluated using standard peptides, proteins, and *in vitro* nitrated whole mouse brain homogenate and shown to provide a significant improvement for the characterization of the nitrotyrosine proteome.

Experimental Procedures

1. Chemicals and Materials. All chemicals, unless specified otherwise, were from Sigma-Aldrich (St. Louis, MO). *N*-Succinimidyl *S*-Acetylthioacetate (SATA), micro-BCA assay kits, and Handee Mini-Spin columns were purchased from Pierce Biotech (Rockford, IL). Thiopropyl sepharose 6B beads were obtained from Amersham Biosciences (Uppsala, Sweden). RapiGest SF reagent (sodium 3-[2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxy]-1-propanesulfonate) was from Waters Corporation (Bedford, MA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). The standard nitrated peptide was purchased from Global Peptide Services (Fort Collins, CO) with 24 u modification on the N-terminus. The characterization of this peptide is in Supporting Information.

2. *In Vitro* Nitration of Model Proteins and Mouse Brain Tissue Homogenate. To nitrate model proteins, human histone 1.2 and bovine serum albumin (BSA), 1 mg (1 mg/mL) of protein in 50 mM PBS (pH 7.4) or NH_4HCO_3 (pH 7.8) was treated with 1 mM peroxynitrite (Calbiochem, San Diego, CA) by bolus addition.[32] After quick vortexing, the sample was incubated for 10 min on ice and then snap-frozen in liquid N_2 until further use.

A C57BL/6J inbred strain male mouse (Jackson Laboratory, Bar Harbor, ME) was sacrificed following deep anesthesia with isoflurane at 9 weeks of age. The whole brain was dissected and then snap-frozen in liquid N_2. To prepare mouse brain homogenate, 50 mM NH_4HCO_3 (pH 7.8) containing 0.5% RapiGest was added to the brain tissue. Tissue homogenization was performed with a pestle type mini-homogenizer (Sigma-Aldrich, St. Louis, MO) three times with 0.5 min duration each time. The sample was kept on ice during the intermittent periods. The resultant homogenates (5 mg/mL, ~10 mg of total proteins) were treated with 2 mM peroxynitrite by bolus

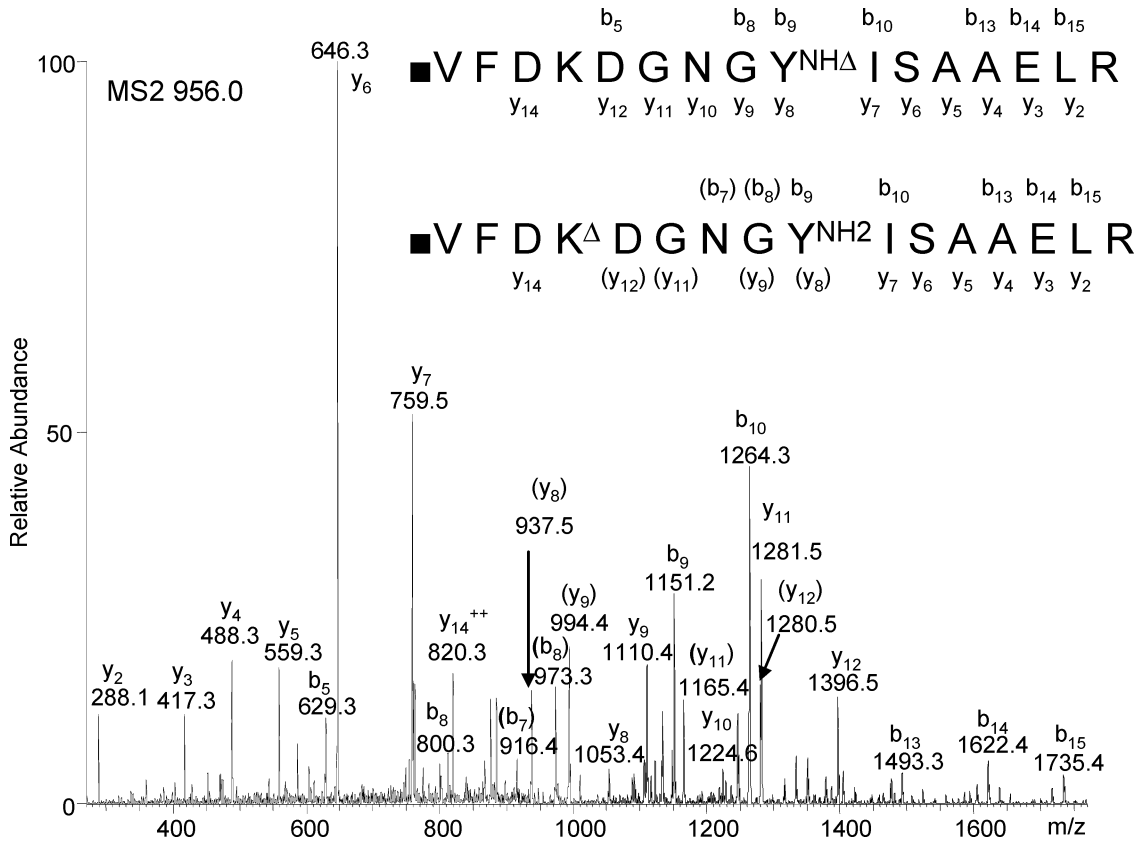


Figure 1. Product ion spectrum for the $[M + 2H]^{2+}$ ions of two different products resulted from SATA reacting with either ϵ-NH_2 on lysine or tyrosyl amino group in model peptide ■VFDKDGNGY^{NH2}ISAAELR. Specific ions corresponding to products originated from the ϵ-NH_2 on lysine are labeled in parenthesis. Δ represents $COCH_2SCOCH_3$. ■ represents the blocked N-terminal amine with a mass increase of 24 u, of which the exact modification type is unclear. The spectrum was acquired on a LCQ-Deca XP instrument (Thermo, San Jose, CA) with direct sample infusion. The origin of ■ is further shown in the Supporting Information.

Table 1. Summary of SATA Reaction with Primary Amines on Tryptic Peptides from BSA Digest[a]

ratio SATA/peptides	pH	total number of K-containing peptides identified	number of peptides containing SATA acetylation of K	percentage of K containing peptides reacted with SATA
100:1	5.0	255	114	45%
100:1	7.0	128	110	86%

[a] The number of peptides shown is peptides/spectra identified by Sequest search after passing our filtering criteria; they are not unique peptides. All peptides have SATA modified N-terminus.

addition. After quick vortexing, the sample was incubated for 10 min on ice and then snap-frozen in liquid N_2 before further processing.

3. Protein Digestion for *in Vitro* Nitrated Protein Samples. RapiGest was added to the *in vitro* nitrated standard proteins for a final concentration of 0.5%. Proteins were denatured by boiling the samples for 5 min and chilled on ice. Disulfide bonds in protein were reduced by 5 mM dithiothreitol (DTT) with 1 h incubation at 37 °C, amd the sulfhydryl groups were alkylated with 20 mM iodoacetamide at room temperature for 1 h in the dark. Samples were then diluted 5-fold with 50 mM NH_4HCO_3 (pH 7.8) to reduce the RapiGest concentration to below 0.1% prior to the addition of sequencing-grade modified trypsin at a ratio of 1:50 (w/w, enzyme/protein). Samples were digested at 37 °C for 3 h with gentle shaking. The RapiGest was decomposed by acidifying the sample with 500 mM HCl at 1/10 volume and incubated for 45 min at 37 °C before centrifuging at 16 000*g* for 10 min to remove the decomposed RapiGest. The cleaned sample was lyophilized in a Speed-Vac (Thermo Savant, Milford, MA).

In vitro nitrated mouse brain homogenate was processed as follows. The protein mixture was denatured with 6 M Guanidine-HCl, and disulfide bonds were reduced with 5 mM tributyl phosphine (TBP) at 45 °C for 1 h followed by addition of 15 mM iodoacetamide. After alkylation of the cysteines, the mixture was diluted 6-fold with 50 mM NH_4HCO_3 (pH 7.8). Trypsin was added at a ratio of 1:50 (enzyme/protein), and the proteins were digested at 37 °C overnight. The final digestion mixture was acidified to remove RapiGest prior to C_{18} SPE cleanup.

4. Acetylation of Primary Amines. The dried tryptic peptides were redissolved in 50 mM $NaHCO_3$ solution (4 mg/mL), and the pH was adjusted to 8.5. Acetic anhydride (totally 200-fold molar excess) in 50 μL of acetonitrile was added to the peptides in three times. The molar excess was calculated with respect to estimated molar quantity of primary amines in peptides, assuming that average MW of each peptide is 1500 and there are one N-termini and 2 lysines in each peptide. Before each addition of acetic anhydride, the pH was readjusted to above 7 with NH_4OH. After the final addition, the sample was incubated at 30 °C for 1 h.[33] To reverse the possible O-

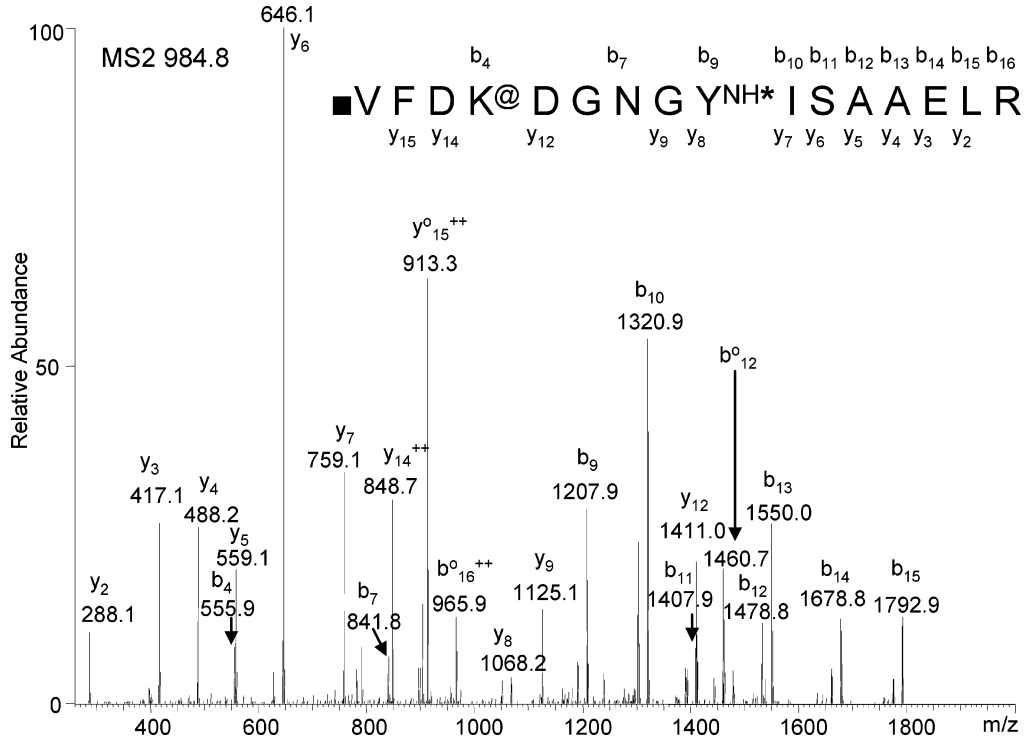


Figure 2. Product ion spectrum for the $[M + 2H]^{2+}$ ion of products resulted from model peptide ■VFDKDGNGY^{NO2}ISAAELR going through all the reactions delineated in Scheme 1. * represents $COCH_2SCH_2CONH_2$, the final modification group on enriched nitrotyrosine; @ represents acetylation, $COCH_3$; ■ represents the blocked N-terminal amine with a mass increase of 24 u. The spectrum was acquired on a LCQ-Deca XP instrument operated in LC−MS/MS mode.

acetylations, hydroxylamine was added at a final concentration of 20 mM and incubated for 20 min.[33] The final reaction mixture was diluted with water to reduce the acetonitrile content to below 5% followed by C_{18} SPE clean up. The eluted peptides from the SPE column were dried in a Speed-Vac.

5. Reduction of Nitrotyrosine to Aminotyrosine. The acetylated peptide samples were redissolved in 50 mM sodium phosphate buffer (4 mg/mL), and the pH was adjusted to 8. Concentrated $Na_2S_2O_4$ solution was added (50-fold molar excess to the expected molar amount of nitrated tyrosines in the sample, assuming that tyrosine natural occurrence in proteins is 3.25% (http://prowl.rockefeller.edu/ aainfo/struct.htm) and 50% of all tyrosines could be nitrated) to reduce the 3-nitrotyrosine to 3-aminotyrosine.[34] The mixture was incubated at room temperature for 20 min followed by C_{18} SPE cleanup to remove the excess reducing agent. The eluted peptides from the SPE column were dried in a Speed-Vac.

6. *S*-Acetylthioacetylation of Aminotyrosine and Deprotection of *S*-Acetyl Group. The above dried peptide residue was redissolved in 100 mM sodium acetate solution (4 mg/mL), and the pH was adjusted to 5. Then SATA/acetonitrile solution was added at a 50-fold molar excess to the expected amount of aminotyrosines, and the mixture was incubated at 37 °C overnight. The reaction mixture was further deprotected with 0.5 M NH_2OH to expose the free sulfhydryls for the following enrichment step. The final mixture was cleaned up by C_{18} SPE column to remove salt and excess reagents. To minimize the oxidation of sulfhydryl groups during the cleanup process, the C_{18} SPE eluting solution (80/20/0.1%, CH_3CN/H_2O/TFA) contained 2 mM EDTA.[35]

7. Enrichment of Sulfhydryl-Derivatized Peptides. The peptides derived from the reduced 3-nitrotyrosine were isolated by a recently reported solid-phase cysteinyl (or sulfhydryl-containing) peptide enrichment approach.[31] Briefly, the peptide residue was redissolved in 100 μL of coupling buffer that consisted of 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, then transferred to a Handee Mini-Spin column that contained ~100 μL of pre-washed thiopropyl sepharose 6B resin. The sulfhydryl-derivatized peptides were captured by the sepharose beads during 1 h incubation at room temperature with gentle shaking. The unbound portion of the peptide sample was removed from the resin by stringent washing; first with the coupling buffer, followed by 2 M NaCl, then 80% acetonitrile/0.1% TFA solution and again with the coupling buffer. The captured sulfhydryl peptides were released by incubating the resin with 100 μL of 20 mM freshly prepared DTT solution at room temperature for 30 min. The released peptides were further alkylated with 40 mM iodoacetamide for 1 h at room temperature in the dark. The alkylated peptide samples were then desalted using C_{18} SPE columns. After concentrating the eluting fraction to a small volume, peptides were subjected to capillary LC−MS/MS analysis.

8. Capillary LC−MS/MS Analysis. The enriched peptides containing derivatized 3-nitrotyrosine were analyzed with automated capillary HPLC systems[36] coupled online with an LTQ ion trap mass spectrometer (ThermoElectron, San Jose, CA) by using an electrospray ionization interface. The reversed-phase capillary columns were prepared by slurry packing 3-μm Jupiter C_{18} particles (Phenomenex, Torrence, CA) into either a 150 μm i.d. × 65 cm or 50 μm i.d. × 65 cm fused silica capillary (Polymicro Technologies, Phoenix, AZ). The mobile phase solvents consisted of (A) 0.2% acetic acid and 0.05% TFA in water and (B) 0.1% TFA in 90% acetonitrile. An exponential gradient was used for the separation, which started with 100% A and gradually increased to 60% B over 100 min. The instrument was operated in data-dependent mode with an m/z

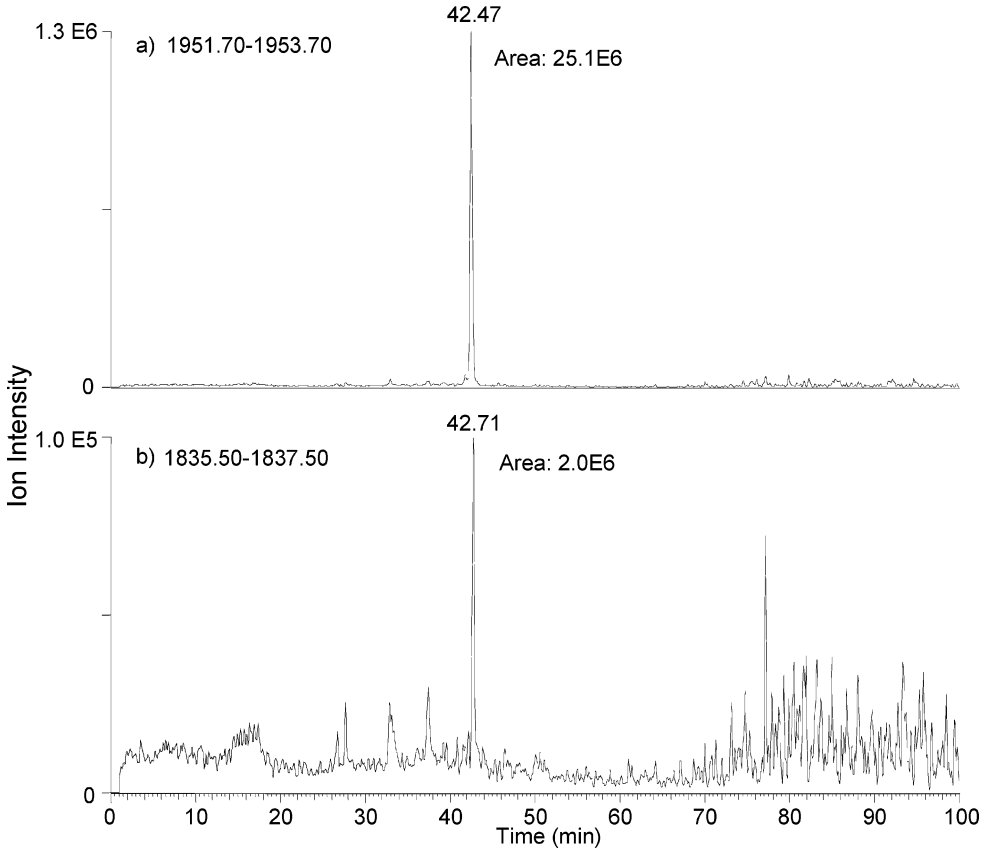


Figure 3. Selected Ion Chromatograms (SICs) of the derivatized product (a) and unreacted starting material (b) for SATA acetylation reaction; the SICs were plotted by selecting mass ranges corresponding to $[M + H]^+ \pm 1$ u, respectively. Product represents ■VFDK@-DGNGY^{NHA}ISAAELR; starting material represents ■VFDK@DGNGY^{NH2}ISAAELR. The symbols are the same as shown in the legend of Figure 1. The identity of each peak was further confirmed by its product ion spectrum (not shown). A LCQ-Deca XP instrument operated in LC−MS/MS mode was used in this study.

Table 2. Summary of the Different Nitrotyrosine-Containing Peptides Identified from *in Vitro* Nitrated Human Histone H1.2 through Our Enrichment Strategy[a]

peptide sequence	XCorr	charge state	spectra counts	protein name
E.RGGVSLAALKKALAAAGY*DVEK@NNSR.I	5.43	3	3	H1.1
K.ALAAAGY*DVEK@NNSR.I	5.29	2	18	H1.2
K.ALAAAGY*DVEKNNSR.I	5.03	2	4	H1.2
K.ERSGVSLAALK@K@ALAAAGY*DVEK@NNSR.I	7.62	3	2	H1.2
K.K@ALAAAGY*DVEK@.N	4.56	2	1	H1.2
K.K@ALAAAGY*DVEK@NNSR.I	5.49	2	9	H1.2
K.K@ALAAAGY*DVEKNNSR.I	4.08	2	2	H1.2
K.KALAAAGY*DVEK@NNSR.I	2.58	2	1	H1.2
R.SGVSLAALK@K@ALAAAGY*DVEK@.N	5.06	3	1	H1.2
R.SGVSLAALK@K@ALAAAGY*DVEK@NNS.R	5.26	3	1	H1.2
R.SGVSLAALK@K@ALAAAGY*DVEK@NNSR.I	6.56	3	8	H1.2
R.SGVSLAALK@K@ALAAAGY*DVEKNNSR.I	6.65	3	2	H1.2
K.ALAAGGY*DVEK@NNSR.I	2.47	2	1	H1.5
K.K@ALAAGGY*DVEK@NNSR.I	4.30	2	1	H1.5

[a] Periods indicate ends of peptides; for internal peptides, the amino acids preceding and following the cleavage site are given. * represents $NHCOCH_2SCH_2CONH_2$, the final modification group on enriched nitrotyrosine; # represents oxidation (O); @ represents acetylation, $COCH_3$; all N-terminal and Cys in the peptides shown were modified with acetylation ($COCH_3$) and alkylation (CH_2CONH_2). respectively.

range of 400−2000. The 10 most abundant ions from each MS scan were selected for further MS/MS analysis by using a normalized collision energy of 35%. Dynamic exclusion of 60 s was applied to avoid repeated analyses of the same abundant precursor ion.

9. Data Analysis. Sequest (ThermoElectron) was used for peptide identification by searching LC−MS/MS data against specific fasta files (BSA fasta file for *in vitro* nitrated BSA sample, and a custom-made human histone database of all family members of human histones for *in vitro* nitrated human histone H1.2 sample) or the mouse International Protein Index (IPI) database (Version 3.09) for the mouse brain homogenate. A parameter which incorporates the following modifications was used in the Sequest search for enriched peptides: static mass modification for cysteinyl residues (carbamidomethylation with iodoacetamide, 57.02 u), static modification for

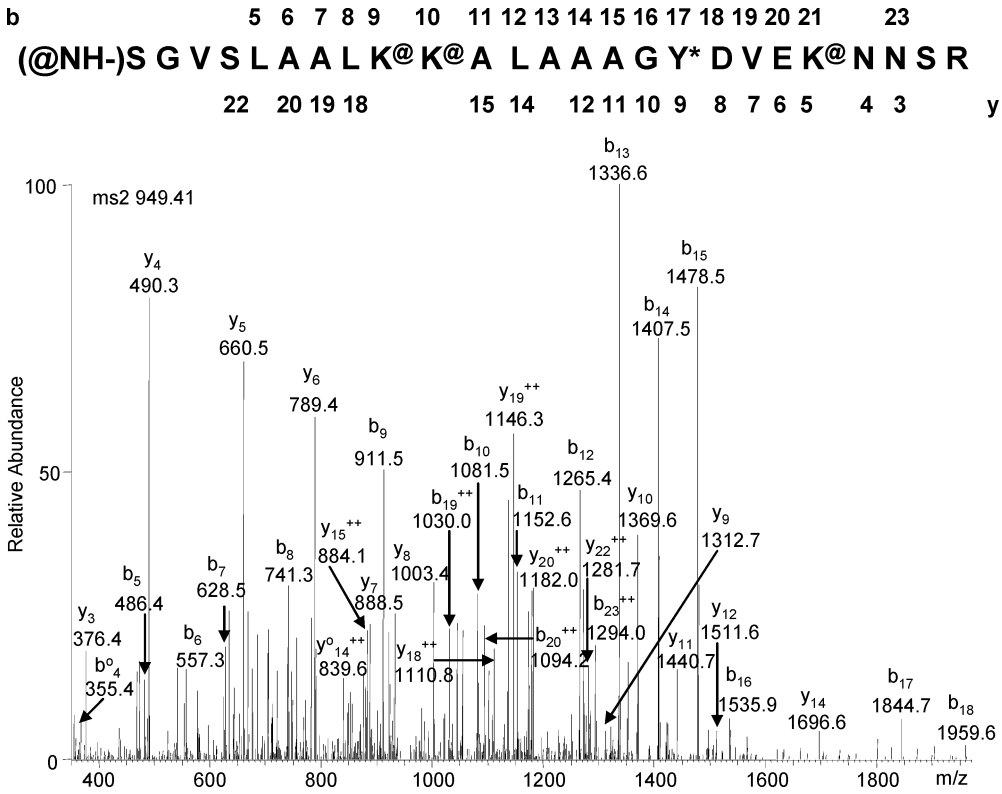


Figure 4. Product ion spectrum for peptide (@NH-)SGVSLAALK@K@ALAA AGY^{NH}*DVEK@NNSR, which is identified from the final enriched peptide mixture of *in vitro* nitrated human histone H1.2. The symbols are the same as shown in the previous Figures.

N-terminal amine (acetylation, 42.01 u), dynamic modification for methionine (oxidation, 16.00 u), dynamic modification for lysine (acetylation, 42.01 u), and dynamic modification for tyrosine (corresponding to the final modification as illustrated in the enrichment scheme, 146.01 u). For comparison purposes, tryptic peptides obtained from *in vitro* nitrated mouse brain homogenate were submitted for global proteomic analysis prior to enrichment. The parameter used for unenriched peptides was as follows: dynamic mass modification for cysteinyl residues (carbamidomethylation with iodoacetamide, 57.02 u), dynamic modification for methionine (oxidation, 16.00 u), and dynamic modification for tyrosine (nitration, 44.99 u). The following criteria used to filter raw Sequest results were established by applying a probability-based evaluation using sequence-reversed database searching as previously described to provide >95% overall confidence level for the entire set of different peptide identifications (<5% false-positive rate),[37] and further confirmed in the mouse brain proteome analysis.[38] (1) Xcorr ≥1.6 for charge state +1 fully tryptic peptides; (2) Xcorr ≥2.4 for charge state +2 fully tryptic peptides and Xcorr ≥4.3 for +2 partially tryptic peptides; and (3) Xcorr ≥3.2 for charge state +3 fully tryptic peptides and Xcorr ≥4.7 for +3 partially tryptic peptides. The delta correlation value (ΔCn) ≥ 0.1 was used in all cases. Additionally, each data set was searched against the reversed mouse IPI database to estimate the False Discovery Rate of the results.

Results and Discussion

Our enrichment strategy (Scheme 1) involves specific derivatization of 3-nitrotyrosine to form free sulfhydryl functional groups, taking advantage of the highly efficient sulfhydryl-containing peptide enrichment method developed in our research group.[31] The derivatization scheme starts with blocking N-terminal α-amine and lysine ϵ-amine in peptides, followed by reducing the nitrotyrosine to aminotyrosine by dithionite. The resulting aminotyrosine is then reacted with SATA, an *N*-hydroxysuccinimide (NHS) ester used for introducing protected sulfhydryl groups into proteins, peptides, and other molecules.[39] As the only available primary amine, the tyrosyl amine reacts with NHS ester to form a stable, covalent amide bond by nucleophilic attack. The free sulfhydryls are formed following the hydroxylamine-mediated deprotection step and can be effectively captured with thiopropyl sepharose beads in the subsequent peptide enrichment step. Captured peptides are eluted by cleaving the new disulfide bond on the resin with a high concentration of DTT.[31] To avoid the competition of native cysteines with the derivatized sulfhydryl groups from 3-nitrotyrosine, samples are reduced and alkylated before

Table 3. Summary of the Different Nitrotyrosine-Containing Peptides Identified from *in Vitro* Nitrated BSA through Our Enrichment Strategy[a]

peptide sequence	XCorr	charge state	modification site
K.Y*ICDNQDTISSK@.L	4.91	2	286
R.RHPEY*AVSVLLR.L	3.60	3	364
K.LGEY*GFQNALIVR.Y	4.19	2	424
R.CCTK@PESERM#PCTEDY*LSLILNR.L	4.78	3	475
R.CCTK@PESERMPCTEDY*LSLILNR.L	3.53	3	475
R.M#PCTEDY*LSLILNR.L	4.03	2	475
R.MPCTEDY*LSLILNR.L	3.96	2	475
R.RPCFSALTPDETY*VPK@.A	4.68	2	520
K.Y*LYEIAR.R (LCQ data)	2.23	1	161

[a] Symbols are the same as in the legend of Table 2.

Table 4. Summary of the Different Nitrotyrosine-Containing Peptides Identified from *in Vitro* Nitrated Mouse Brain through Our Enrichment Strategy[a]

IPI no.	gene	protein name	nitrated peptide sequence	XCorr	charge
IPI00309068.3	Zbtb33	Kaiso Protein, Full Insert Sequence	R.M#K@VK@HDDHYELIVDGRVY*Y*ICIVCK@R.S	3.21	3
IPI00116498.1	Ywhaz	14-3-3 Protein Zeta/Delta	R.NLLSVAY*K@NVVGAR.R	3.26	2
IPI00116498.1	Ywhaz	14-3-3 Protein Zeta/Delta	K.TAFDEAIAELDTLSEESY*K@DSTLIM#QLLR.D	5.79	3
IPI00116498.1	Ywhaz	14-3-3 Protein Zeta/Delta	K.TAFDEAIAELDTLSEESY*K@.D	2.87	2
IPI00408378.4	Ywhaq	14-3-3 Protein Theta	K.TAFDEAIAELDTLNEDSY*K@DSTLIM#QLLR.D	3.90	3
IPI00230707.5	Ywhag	14-3-3 Protein Gamma	K.TAFDDAIAELDTLNEDSY*K@DSTLIM#QLLR.D	4.26	3
IPI00118384.1	Ywhae	14-3-3 Protein Epsilon	K.AAFDDAIAELDTLSEESY*K@DSTLIMQLLR.D	4.48	3
IPI00118384.1	Ywhae	14-3-3 Protein Epsilon	K.AAFDDAIAELDTLSEESY*K@DSTLIM#QLLR.D	6.10	3
IPI00118384.1	Ywhae	14-3-3 Protein Epsilon	K.AAFDDAIAELDTLSEESY*K@.D	2.86	2
IPI00230682.5	Ywhab	14-3-3 Protein Beta/Alpha	K.TAFDEAIAELDTLNEESY*K@DSTLIM#QLLR.D	4.18	3
IPI00132347.1	Uqcrb	Ubiquinol-Cytochrome C Reductase Binding Protein	R.RLPEDLY*NDR.M	2.57	2
IPI00109073.4	Tubb4	Tubulin Beta4 Chain	K.FWEVISDEHGIDPTGTY*HGDSDLQLER.I	5.55	3
IPI00109073.4	Tubb4	Tubulin Beta4 Chain	K.GHY*TEGAELVDAVLDVVR.K	6.10	2
IPI00109073.4	Tubb4	Tubulin Beta4 Chain	K.GHY*TEGAELVDAVLDVVRK@.E	4.74	2
IPI00112251.1	Tubb3	Tubulin Beta 3 Chain	K.LATPTY*GDLNHLVSATM#SGVTTSLR.F	4.01	2
IPI00338039.1	Tubb2	Tubulin Beta 2	R.INVYY*NEAAGNK@YVPR.A	2.97	2
IPI00117350.1	Tuba4	Tubulin Alpha 4 Chain	R.GHY*TIGK@EIIDPVLDR.I	2.90	2
IPI00110753.1	Tuba1	Tubulin Alpha 1 Chain	R.IHFPLATY*APVISAEK@.A	4.11	1
IPI00110753.1	Tuba1	Tubulin Alpha 1 Chain	K.VGINY*QPPTVVPGGDLAK@.V	2.96	2
IPI00110753.1	Tuba1	Tubulin Alpha 1 Chain	R.IHFPLATY*APVISAEK.A	3.35	2
IPI00110753.1	Tuba1	Tubulin Alpha 1 Chain	R.NLDIERPTY*TNLNR.L	3.44	2
IPI00110753.1	Tuba1	Tubulin Alpha 1 Chain	R.QLFHPEQLITGK@EDAANNY*AR.G	6.33	2
IPI00604879.1	Tuba1	Tubulin Alpha 1	R.IHFPLATY*SPVISAEK@.A	3.88	2
IPI00110753.1	Tuba1	Tubulin Alpha 1 Chain	R.FDGALNVDLTEFQTNLVPY*PR.I	3.68	2
IPI00467833.4	Tpi1	Triosephosphate Isomerase	K.VVLAY*EPVWAIGTGK@.T	3.78	2
IPI00137409.3	Tkt	Transketolase	K.NM#AEQIIQEIY*SQVQSK@.K	2.76	2
IPI00118413.2	Thbs1	Thrombospondin 1	K.TGY*IRVVMY*EGK@K@IM#ADSGPIYDKTYAGGR.L	3.23	3
IPI00137368.4	Spna2	Predicted: Spectrin Alpha 2	K.NQALNTDNY*GHDLASVQALQR.K	3.44	2
IPI00137368.4	Spna2	Predicted: Spectrin Alpha 2	K.K@HEALMSDLSAY*GSSIQALR.E	3.03	2
IPI00137368.4	Spna2	Predicted: Spectrin Alpha 2	K.IAALQAFADQLIAVDHY*AK@.G	4.24	2
IPI00115157.1	Snca	Alpha Synuclein	K.TK@EGVLY*VGSK@.T	3.11	2
IPI00308162.3	Slc25a12	Calcium Binding Mitochondrial Carrier Protein Aralar1	R.IAPLAEGALPY*NLAELQR.Q	3.30	2
IPI00112536.1	Scrn1	Secernin-1	R.SSPCIHY*FTGTPDPSR.S	2.83	2
IPI00222557.4	S100b	S-100 Calcium Binding Protein Beta Subunit	K.AMVALIDVFHQY*SGR.E	4.13	2
IPI00395193.1	Rtn1	Reticulon 1	K.HQAQVDQY*LGLVR.T	3.54	2
IPI00227069.1	Ppm1h	Hypothetical Protein Phosphatase 2C Domain Containing Protein	R.SAY*NISGGCTALIVVCLLGKLYVANAGDSRAIIIR.N	3.34	3
IPI00407130.3	Pkm2	Pyruvate Kinase 3	R.EATESFASDPILY*RPVAVALDTK@.G	3.59	3
IPI00407130.3	Pkm2	Pyruvate Kinase 3	R.EATESFASDPILY*RPVAVALDTK@GPEIR.T	4.25	3
IPI00230002.4	Pgk1	PGK1 Protein	K.SVVLMSHLGRPDGVPM#PDK@Y*SLEPVAAELK@.S	3.39	3
IPI00230002.4	Pgk1	PGK1 Protein	K.SVVLM#SHLGRPDGVPM#PDK@Y*SLEPVAAELK@.S	3.30	3
IPI00230002.4	Pgk1	PGK1 Protein	K.LGDVY*VNDAFGTAHR.A	3.35	2
IPI00230706.4	Pgam2	Phosphoglycerate Mutase 2	R.HY*GGLTGLNK@AETAAK@.H	2.71	2
IPI00123613.1	Pacsin1	Protein Kinase C and Casein Kinase Substrate in Neurons Protein 1	R.GRLDSGQLGLYPANY*VEAI.-	3.94	2
IPI00117657.1	Opa1	Dynamin Like 120 KDa Protein, Mitochondrial Precursor	K.VNDEHPAY*LASDEITTVR.K	3.28	2
IPI00114241.1	Nefh	Neurofilament Triplet H Protein	R.HQADIASY*QDAIQQLDSELR.N	3.91	2
IPI00323800.4	Nef3	Neurofilament Triplet M Protein	K.QASHAQLGDAY*DQEIR.E	4.62	2
IPI00109167.1	Ndufv2	NADH Ubiquinone Oxidoreductase 24 KDa Subunit, Mitochondrial Precursor	K.NY*PEGHQAAAVLPVLDLAQR.Q	3.21	2
IPI00169925.1	Ndufv2	NDUFV2 Protein	R.IEAIVK@NY*PEGHQAAAVLPVLDLAQR.Q	3.14	2
IPI00308882.4	Ndufs1	NADH Ubiquinone Oxidoreductase 75 KDa Subunit, Mitochondrial Precursor	R.ALSEIAGITLPY*DTLDQVR.N	2.68	2
IPI00136134.1	Ndrg2	NDRG2 Protein	K.RPAIFTY*HDVGLNYK@.S	3.46	2
IPI00323592.1	Mdh2	Malate Dehydrogenase, Mitochondrial Precursor	R.LTLY*DIAHTPGVAADLSHIETR.A	6.84	3
IPI00323592.1	Mdh2	Malate Dehydrogenase, Mitochondrial Precursor	K.HGVY*NPNK@IFGVTTLDIVR.A	3.35	2
IPI00115240.1	Mbp	Splice Isoform 1 of Myelin Basic Protein	R.TTHY*GSLPQK@SQHGR.T	4.00	2
IPI00115240.1	Mbp	Splice Isoform 1 of Myelin Basic Protein	R.TTHY*GSLPQK@.S	2.61	1
IPI00115240.1	Mbp	Splice Isoform 1 of Myelin Basic Protein	R.SK@Y*LATASTM#DHAR.H	3.00	2
IPI00115240.1	Mbp	Splice Isoform 1 of Myelin Basic Protein	K.Y*LATASTMDHAR.H	2.61	2
IPI00115240.1	Mbp	Splice Isoform 1 of Myelin Basic Protein	K.Y*LATASTM#DHAR.H	3.60	2
IPI00113584.2	Mapt	Splice Isoform PNS-Tau of Microtubule associated protein Tau	K.HVPGGGSVQIVY*K@PVDLSK@.V	3.31	2
IPI00458204.1	LOC433839	Predicted: Similar to Tubulin Beta 2	K.FWEVISDEHGIDTTGTY*HGDSDLQLER.I	4.30	3
IPI00606203.1	LOC433410	Predicted: Similar to Heterogeneous Nuclear Ribonucleoprotein A1	R.SSGPYGGGGQY*IAK@PGNQGGYGGSSSSSSSYGSGR.R	3.27	3

Table 4. (Continued)

IPI no.	gene	protein name	nitrated peptide sequence	XCorr	charge
IPI00112366.3	LOC243823	Predicted: Similar to ISOC2 Protein	R.LLDVPILLTEQY*PEGLGPTVPELGAQGIRPVSK@.T	4.76	3
IPI00336741.2	LOC238558	HIST1H4I Protein (Fragment)	R.K@TVTAMDVVY*ALK@R.Q	2.66	2
IPI00336741.2	LOC238558	HIST1H4I Protein (Fragment)	R.K@TVTAM#DVVY*ALK@R.Q	3.10	2
IPI00329998.2	LOC238558	Histone 4 Protein, Full Insert Sequence	R.DAVTY*TEHAK@R.E	2.49	2
IPI00329998.2	LOC238558	Histone 4 Protein, Full Insert Sequence	K.VFLENVIRDAVTY*TEHAK@.R	4.20	3
IPI00126917.1	LOC237880	Predicted: Similar to Prohibitin	R.K@LEAAEDIAY*QLSR.S	3.03	2
IPI00229080.4	Hspcb	Heat Shock Protein 84B	K.VILHLK@EDQTEY*LEER.R	4.16	2
IPI00330804.3	Hspca	Heat Shock Protein HSP 90-Alpha	K.HSQFIGY*PITLFVEK@.E	3.19	2
IPI00323357.1	Hspa8	Heat Shock Cognate 71 KDa Protein	K.TVTNAVVTVPAY*FNDSQR.Q	4.02	2
IPI00323357.1	Hspa8	Heat Shock Cognate 71 KDa Protein	K.SINPDEAVAY*GAAVQAAILSGDK@.S	3.08	2
IPI00323357.1	Hspa8	Heat Shock Cognate 71 KDa Protein	R.IINEPTAAAIAY*GLDK@K@.V	2.44	2
IPI00319992.1	Hspa5	78 KDa Glucose Regulated Protein Precursor	K.VTHAVVTVPAY*FNDAQR.Q	3.79	2
IPI00319992.1	Hspa5	78 KDa Glucose Regulated Protein Precursor	K.K@VTHAVVTVPAY*FNDAQR.Q	2.77	2
IPI00133208.3	Hspa1l	Heat Shock 70 KDa Protein 1L	R.IINEPTAAAIAY*GLDK@.G	4.25	2
IPI00123794.3	Hspa1a	Heat Shock Protein 1A	R.TTPSY*VAFTDTER.L	3.28	2
IPI00315227.5	Hnrpa2b1	Heterogeneous Nuclear Ribonucleoproteins A2/B1	K.Y*HTINGHNAEVR.K	2.87	2
IPI00230730.3	Hist2h3c2	Histone H3.2.	R.Y*RPGTVALR.E	2.63	2
IPI00223713.4	Hist1h1c	Histone H1.2.	K.ALAAAGY*DVEK@NNSR.I	2.73	2
IPI00273806.1	Hecw2	Hypothetical C2 Domain /C2-Domain Profile Containing Protein	K.LVSFTLSDLRAVGLKKGM#FFNPDPY*LK@M#SIQPGK@.K	3.40	3
IPI00126208.1	Hbb-b1	Hemoglobin Beta Adult Major Chain, Full insert Sequence	K.VVAGVAAALAHK@Y*H.-	4.75	2
IPI00110658.1	Hba-a1	Hemoglobin Beta Adult Major Chain, Full insert Sequence	K.TY*FPHFDVSHGSAQVK@.G	3.25	2
IPI00110658.1	Hba-a1	Hemoglobin Beta Adult Major Chain, Full insert Sequence	K.IGGHGAEY*GAEALER.M	3.69	2
IPI00230212.4	Gstm1	Glutathione S-Transferase MU1	K.VTYVDFLAY*DILDQYR.M	2.58	2
IPI00121515.2	Gpiap1	Cytoplasmic Activation Proliferation Associated Protein 1	K.TVLELQY*VLDK@LGDDDVR.T	2.47	2
IPI00117312.1	Got2	Aspartate Aminotransferase, Mitochondrial Precursor	K.ILIRPLY*SNPPLNGAR.I	2.84	2
IPI00117312.1	Got2	Aspartate Aminotransferase, Mitochondrial Precursor	K.NLDK@EY*LPIGGLAEFCK@.A	2.62	2
IPI00230204.6	Got1	Aspartate Aminotransferase, Cytoplasmic	K.IANDNSLNHEY*LPILGLAEFR.S	3.11	2
IPI00115544.1	Gng4	Guanine Nucleotide Binding Protein G(I)/G(S)/G(O) Gamma 4 Subunit	K.VSQAASDLLAY*CEAHVR.E	3.13	2
IPI00467152.2	Gnai1	Predicted: Guanine Nucleotide Binding Protein, Alpha Inhibiting 1	R.IAQPNY*IPTQQDVLR.T	2.53	2
IPI00127207.1	Glul	Glutamine Synthetase	R.TCLLNETGDEPFQY*K@N.-	3.15	2
IPI00127207.1	Glul	Glutamine Synthetase	R.RPSANCDPY*AVTEAIVR.T	4.26	2
IPI00122565.1	Gdi3	Splice Isoform 1 of RAB GDP Dissociation Inhibitor Beta 2	K.SPYLYPLY*GLGELPQGFAR.L	2.57	2
IPI00129928.2	Fh1	Fumarate Hydratase, Mitochondrial Precursor	R.IY*ELAAGGTAVGTGLNTR.I	3.31	2
IPI00116753.3	Etfa	Electron Transfer FlavoProtein Alpha Subunit, Mitochondrial Precursor	K.LLY*DLADQLHAAVGASR.A	4.65	2
IPI00122684.1	Eno2	ENO2 Protein (Fragment)	R.AAVPSGASTGIY*EALELR.D	3.29	2
IPI00122684.1	Eno2	ENO2 Protein (Fragment)	K.M#VIGM#DVAASEFY*R.D	3.26	2
IPI00122684.1	Eno2	ENO2 Protein (Fragment)	R.Y*ITGDQLGALYQDFVR.N	3.72	2
IPI00323385.1	Dyrk1b	Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1B	K.GSFGQVVKAY*DHQTQELVAIKIIKNK@K@.A	3.37	3
IPI00114375.1	Dpysl2	Dihydropyrimidinase Related Protein 2	R.FQLTDSQIY*EVLSVIR.D	3.41	2
IPI00114375.1	Dpysl2	Dihydropyrimidinase Related Protein 2	K.THNSALEY*NIFEGM#ECR.G	5.12	2
IPI00172221.2	Dnm1l	Splice Isoform 2 of Dynamin 1 Like Protein	R.IIQHCSNY*STQELLR.F	2.75	2
IPI00272878.4	Dnm1	Splice Isoform 1 of Dynamin 1	R.K@FFLSHPSY*R.H	3.07	2
IPI00272878.4	Dnm1	Splice Isoform 1 of Dynamin 1	R.IEGSGDQIDTY*ELSGGAR.I	2.69	2
IPI00121623.1	Dnclc1	Dynein Light Chain 1, Cytoplasmic	K.Y*NPTWHCIVGR.N	3.10	2
IPI00119876.1	Dnchc1	Dynein Heavy Chain, Cytosolic	R.FGNPLLVQDVESY*DPVLNPVLNR.E	3.54	2
IPI00113141.1	Cs	Citrate Synthase, Mitochondrial Precursor	R.VVPGY*GHAVLR.K	2.79	2
IPI00117978.1	Cox4i1	Cytochrome C Oxidase Subunit IV Isoform 1, Mitochondrial Precursor	R.DY*PLPDVAHVTM#LSASQK@.A	3.54	2
IPI00169916.9	Cltc	Clathrin Heavy Chain	K.RDPHLACVAY*ER.G	2.42	2
IPI00169916.9	Cltc	Clathrin Heavy Chain	R.LASTLVHLGEY*QAAVDGAR.K	4.99	2
IPI00114409.1	Clta	Clathrin Light Chain A	K.AIK@ELEEWY*AR.Q	2.82	2
IPI00120076.2	Ckmt2	Creatine Kinase, Sarcomeric Mitochondrial Precursor	R.LGY*ILTCPSNLGTGLR.A	4.29	2
IPI00128296.1	Ckmt1	Creatine Kinase, Ubiquitous Mitochondrial Precursor	K.TVGM#VAGDEETY*EVFAELFDPVIQER.H	4.62	3

Table 4. (Continued)

IPI no.	gene	protein name	nitrated peptide sequence	XCorr	charge
IPI00136703.1	Ckb	Creatine Kinase B-Type	K.VLTPELY*AELR.A	3.51	2
IPI00407543.2	Cfl1	Cofilin 1, non-muscle, full insert sequence	K.NIILEEGK@EILVGDVGQTVDDPY*TTFVK@.M	4.96	3
IPI00125182.1	Bmp1	Bone Morphogenetic Protein 1 Precursor	R.CEAACGGFLTK@LNGSITSPGWPK@EY*PPNK@.N	3.34	3
IPI00311461.1	Atp6v1h	Vacuolar ATP Synthase Subunit H (EC 3.6.3.14)	K.LLEVSDDPQVLAVAAHDVGEY*VR.H	2.95	2
IPI00119113.1	Atp6v1b2	Vacuolar ATP Synthase Subunit B, Brain Isoform	K.RIPQSTLSEFY*PR.D	2.95	2
IPI00119113.1	Atp6v1b2	Vacuolar ATP Synthase Subunit B, Brain Isoform	R.Y*AEIVHLTLPDGTK@R.S	2.58	2
IPI00119112.1	Atp6v1a1	Vacuolar ATP Synthase Catalytic Subunit A	K.IK@ADY*AQLLEDMQNAFR.S	4.29	2
IPI00118986.1	Atp5o	ATP Synthase Oligomycin Sensitivity Conferral Protein, Mitochondrial Precursor	K.LVRPPVQVY*GIEGR.Y	2.58	2
IPI00468481.2	Atp5b	ATP Synthase Beta Chain, Mitochondrial Precursor	K.VALVY*GQM#NEPPGAR.A	3.17	2
IPI00468481.2	Atp5b	ATP Synthase Beta Chain, Mitochondrial Precursor	K.GFQQILAGEYDHLPEQAFY*M#VGPIEEAVAK@.A	4.30	3
IPI00468481.2	Atp5b	ATP Synthase Beta Chain, Mitochondrial Precursor	R.AIAELGIY*PAVDPLDSTSR.I	3.19	2
IPI00468481.2	Atp5b	ATP Synthase Beta Chain, Mitochondrial Precursor	R.TREGNDLY*HEM#IESGVINLK@.D	2.97	2
IPI00468481.2	Atp5b	ATP Synthase Beta Chain, Mitochondrial Precursor	R.IMDPNIVGNEHY*DVAR.G	3.70	2
IPI00468481.2	Atp5b	ATP Synthase Beta Chain, Mitochondrial Precursor	R.IM#DPNIVGNEHY*DVAR.G	4.31	2
IPI00130280.1	Atp5a1	ATP Synthase Alpha Chain, Mitochondrial Precursor	R.EAY*PGDVFYLHSR.L	2.40	2
IPI00122048.1	Atp1a3	Na+/K+ ATPase Alpha3 Subunit	R.K@Y*NTDCVQGLTHSK@.A	2.84	2
IPI00122048.1	Atp1a3	Na+/K+ ATPase Alpha3 Subunit	K.Y*NTDCVQGLTHSK@.A	3.02	2
IPI00122048.1	Atp1a3	Na+/K+ ATPase Alpha3 Subunit	K.EQPLDEEMK@EAFQNAY*LELGGLGER.V	3.25	2
IPI00311682.5	Atp1a1	Na+/K+ Transporting ATPase Alpha1 Chain Precursor	K.EQPLDEELK@DAFQNAY*LELGGLGER.V	4.13	2
IPI00119689.1	Ap2b1	Splice Isoform 1 of Adapter Related Protein Complex 2 Beta 1 Subunit	K.LAPPLVTLLSGEPEVQY*VALR.N	2.55	2
IPI00221402.6	Aldoa-ps2	Fructose Bisphosphate Aldolase A	-.PHPY*PALTPEQK@.K	2.91	2
IPI00110850.1	Actb	Actin Cytoplasmic 1	R.GY*SFTTTAER.E	2.34	1
IPI00110850.1	Actb	Actin Cytoplasmic 1	R.TTGIVM#DSGDGVTHTVPIYEGY*ALPHAILR.L	4.36	3
IPI00110827.1	Acta1	Actin, Alpha Skeletal Muscle	K.SY*ELPDGQVITIGNER.F	2.35	1
IPI00110827.1	Acta1	Actin, Alpha Skeletal Muscle	K.IWHHTFY*NELR.V	2.63	2
IPI00116074.1	Aco2	Aconitate Hydratase, Mitochondrial Precursor	K.IVY*GHLDDPANQEIER.G	5.43	2
IPI00154054.1	Acat1	Acetyl CoA Acetyltransferase, Mitochondrial Precursor	R.IAAFADAAVDPIDFPLAPAY*AVPK@.V	2.62	2
IPI00227445.1	Abat	Splice Isoform 2 of 4-aminobutyrate Aminotransferase, Mitochondrial Precursor	K.TLLTGLLDLQAQY*PQFISR.V	2.90	2
IPI00109061.1	2410129E14Rik	Tubulin Beta4 Chain Homologue	K.GHY*TEGAELVDSVLDVVRK@.E	5.80	2
IPI00109061.1	2410129E14Rik	Tubulin Beta4 Chain Homologue	K.GHY*TEGAELVDSVLDVVR.K	6.75	2
IPI00109061.1	2410129E14Rik	Tubulin Beta4 Chain Homologue	K.FWEVISDEHGIDPTGSY*HGDSDLQLER.I	6.09	3
IPI00109061.1	2410129E14Rik	Tubulin Beta4 Chain Homologue	K.LTTPTY*GDLNHLVSATMSGVTTCLR.F	4.08	2
IPI00109061.1	2410129E14Rik	Tubulin Beta4 Chain Homologue	K.LTTPTY*GDLNHLVSATM#SGVTTCLR.F	3.93	3
IPI00112184.1	1700127D06Rik	Similar to Tissue Kallikrein (EC 3.4.21.35), Submandibular MGK-2	R.ERIY*TKLIKFTSWIKDTM#GK@K@TK@TK@.T	3.20	3
IPI00330472.1	0710005I19Rik	RIKEN CDNA 0710005I19	K.M#VSLGTLSY*VLLDAEATKNK.T	2.57	2
IPI00120532.1		21 KDa Protein	K.GNDISSGTVLSDY*VGSGPPSGTGLHR.Y	3.42	2
IPI00130286.2		Glucocorticoid Receptor DNA Binding Factor 1 (Fragment)	R.SFIM#NEDFYQWLEESVYM#DIY*GK@.H	3.28	3
IPI00123176.1		37 KDa Protein	K.LISWYDNEY*GYSNR.M	2.73	2

[a] Symbols are the same as in the legends of Tables 2 and 3.

digestion, which makes the newly generated sulfhydryl group on the tyrosine the only one available in the enrichment step.

Primary amines, that is, N-terminal α- and lysine ϵ-amines, have pK_a values of ∼8 and ∼9.5, respectively. On the other hand, the pK_a of the $-NH_2$ in aminotyrosine is ∼5. In a previous report, it was suggested that amine derivatization reaction can be directed specifically at aminotyrosine if pH was 5.[30] To examine the relative reactivities of native peptide amines and aminotyrosines, a standard peptide containing lysine and aminotyrosine was used to react with SATA at pH 5. It was found that both of the amines on lysine and aminotyrosine can be reactive, as shown in Figure 1 of the product ion spectrum from the same $[M + 2H]^{2+}$ ions of the two different SATA modification products, in which specific ions (expected b_4-b_8 and y_8-y_{12} ions) corresponding to SATA modification either on lysine or on aminotyrosine were identified. Primary amines susceptible to SATA reaction at pH 5 were further confirmed by reaction of SATA with other primary amine-containing peptides as well as a tryptic peptide mixture from BSA protein (Table 1). These results clearly show that α- and ϵ- primary amines are highly reactive with SATA, forming partially or fully SATA-acetylated products. At pH 5 a total of 45% of lysine-containing peptides reacted with SATA. Therefore, these results demonstrate that the specificity required for enrichment of nitrotyrosine is not provided by direct labeling of aminotyrosine with SATA in the presence of α- and ϵ-amino

groups, thus, validating our strategy (Scheme 1) to block all the α- and ϵ-amines in peptides before reduction of nitrotyrosine to aminotyrosine, making aminotyrosine the only reactive amine to SATA.

The primary amine blocking strategy was initially tested with a standard peptide containing nitrotyrosine. This model peptide represents a portion of the sequence of calmodulin, which contains Y^{99}, a nitration-sensitive site.[40] After acetylation with acetic anhydride to block the amino group on lysine, reduction of nitrotyrosine to aminotyrosine, derivatization of the aminotyrosine with SATA, deprotection to expose the free sulfhydryl group, and alkylation of the new sulfhydryl with iodoacetamide, the final peptide was confirmed unambiguously by its product ion spectrum (Figure 2). An important feature about the final product is that the derivatized nitrotyrosine group is relatively stable under the CID conditions used in the LC−MS/MS experiment, aiding peptide identification. As shown, almost complete series of abundant b- and y-ions carrying the modification site are observed with no obvious evidence of labile cleavage products from the modification site. Similar results have also been observed in peptides when aminotyrosine and primary amines were all acetylated with *N*-hydroxysuccinimide acetate,[41] in which it was reported that acetylation of aminotyrosine can enhance peptide backbone cleavage under CID conditions using ion-trap mass spectrometers. We further estimated the efficiency of each reaction step by plotting the selected ion chromatograms for the expected products and underivatized starting materials, taking into account all the charged ions for each species and comparing the peak areas. After optimization of the reaction conditions, we were able to achieve more than 90% efficiency for each reaction step. The efficiency of each chemical reaction is labeled in Scheme 1. The selected ion chromatograms for the SATA acetylation of aminotyrosine step are shown in Figure 3.

We next applied this strategy to the enrichment of nitrotyrosine peptides derived from *in vitro* nitrated proteins. We first tested this approach with *in vitro* nitrated human histone H1.2 which contains only a single tyrosine residue. In addition, the lysine content in histone H1.2 is about 28%, which poses a challenging test for this enrichment method, especially with respect to the first acetylation step for blocking primary amines. Therefore, 200 μg of nitrated histone H1.2 was subjected to the reaction steps in Scheme 1. A single LC−MS/MS analysis provided a total of 59 identified spectra with 54 (91%) corresponding to peptides containing nitrotyrosine; in comparison, the analysis for the unenriched sample had only 9% of the identified spectra contained nitrotyrosine. The 5 non-nitrated peptides are from histone H1.2 and other histone-associated proteins. The 14 different nitrotyrosine peptides are shown in Table 2; most of the multiple nitrotyrosine peptides were the result of different tryptic cleavage sites. In addition to the one nitration site identified from histone H1.2, three additional nitrated peptides are identified from histone H1.1 and H1.5 which are possible sample impurities. A representative product ion spectrum is shown in Figure 4 for a relatively long 25 residue peptide containing multiple modifications, but the product ion spectrum quality is high enough for unambiguous identification of the tyrosine modification site along with the entire peptide sequence. Of note is the high efficiency of primary amine acetylation in which all of the N-termini and the majority of the lysines were acetylated.

To further test the method for enrichment of peptides from proteins with multiple nitrated tyrosine sites, BSA was chosen since this nitration-sensitive protein contains 21 tyrosine sites that can potentially be nitrated *in vitro*. We therefore applied this enrichment strategy to peptides from 200 μg of nitrated BSA. Sixty-five spectra were identified within one LC−MS/MS analysis, 40 (62%) of which contained derivatized nitrotyrosine peptides (compared to only 9% of identified spectra containing nitrotyrosine for the unenriched sample). From these 40 spectra, 8 different peptides corresponding to 5 nitrated sites (Y^{286}, Y^{364}, Y^{424}, Y^{475}, and Y^{520}) were identified as shown in Table 3. *In vitro* nitration of Y^{364}, Y^{424}, and Y^{475} has been reported previously by precursor ion scanning for specific nitrotyrosine immonium ions combined with ESI−MS/MS.[26] Most recently, Y^{286} was also identified by acetylation of aminotyrosines prior to ESI−MS/MS,[41] though Y^{520} has never been previously reported. Y^{161} is also known to be a common site of nitration,[26] but we did not detect this peptide in our initial linear ion trap (LTQ) analysis; however, the nitration of this site was confirmed in a reanalysis with a conventional ion trap (LCQ) LC−MS/MS. It needs to be pointed out that, although 6 tyrosine sites have been identified from *in vitro* nitrated BSA, our data shows that only a portion of the total number of tyrosines in BSA will be nitrated *in vitro*. This is in agreement with the well-documented site selectivity of nitration as previously published from *in vitro* nitration studies for BSA[26,41] as well as other proteins.[42,43]

Finally, we extended our enrichment approach to more complex samples to identify possible nitrated peptides/proteins from *in vitro* nitrated mouse brain tissue homogenate, using 3.1 mg of digested peptides for the enrichment experiment. The final enriched peptides were subjected to duplicate LC−(LTQ)MS/MS analyses. A total of 150 different nitrotyrosine peptides are confidently identified from this sample, corresponding to 102 unique proteins (Table 4). The false discovery rate is estimated to be 3.3% based upon a reverse database search. Many of the identified nitrotyrosine containing proteins have been previously reported as endogenously nitrated. For example, low levels of nitrated 78 kDa glucose-regulated protein precursor, aspartate aminotransferase, and creatine kinase were found in our previously endogenous nitration study of mouse brain,[6] while acetyl-CoA acetyl transferase, ATP synthase, electron-transfer flavoprotein alpha-subunit, and malate dehydrogenase were found endogenously nitrated in muscle.[23]

Approximately 35% of the identified spectra (422 different peptides) contain derivatized nitrotyrosine. A majority (>60%) of the identified non-nitrated peptides are from different proteins and were carried along during the enrichment process (listed as Supporting Information). In the global analysis of the tryptic peptides without enrichment, only about 5.9% identified peptides contain nitrotyrosine (156 of the 2618 different peptides identified from duplicate analyses contain nitrotyrosine). The total number of peptide identifications from the enriched samples has been largely limited by the LC−MS/MS platform sensitivity due to the small quantity of the enriched sample obtained from this experiment, while the global analysis is not limited by the sample quantity. Following further optimization of enrichment recovery and the use of a higher sensitivity LC−MS/MS platform, we anticipate the enrichment strategy should allow more low-abundance nitrotyrosine peptides to be identified. Also notable is that only 17.6% of identified tyrosine-containing peptides from global analysis were observed to be nitrated *in vitro*, which is consistent with *in vitro* nitration of BSA both in this work and by others.[26,41]

This observation also agrees with a previous report that suggested that *in vitro* tyrosine nitration was localized frequently on the protein surface and also depended on neighboring amino acids.[26,32] Therefore, the nitrated peptides and proteins identified in the *in vitro* nitrated brain (listed in Table 4) provide evidence as to which proteins and tyrosine sites are more sensitive to oxidation.

Conclusion and Perspective

A new nitrotyrosine enrichment strategy has been developed by prior blocking of all primary amines, the derivatization of nitrotyrosine to form free sulfhydryls, and solid-phase high efficiency capture/release of derived sulfhydryl-containing peptides. The efficiency of this approach was demonstrated with standard peptides containing nitrotyrosine, *in vitro* nitrated model proteins histone H1.2 and BSA, and *in vitro* nitrated mouse brain tissue homogenate. To our knowledge, this method represents the first demonstration of nitrotyrosine peptide enrichment and identification from a complex global proteome sample.

Furthermore, this enrichment scheme can also be easily coupled with stable isotope labeling techniques such as ^{18}O-labeling for quantitative tyrosine nitration analysis among different biological conditions.[44,45]

One limitation of this approach is the involvement of multiple steps of derivatization and SPE cleanup steps at the peptide level, which can lead to significant sample loss. We did not apply this derivatization strategy at the protein level since some nitration sites might not be easily accessible under the less-than-optimal denaturing conditions that were used in our chemical derivatization steps. However, working at the protein level may provide better sample recovery by potentially separating unwanted salts/excessive reagents from proteins based on size through the use of various membrane-based molecular weight cutoff filters, which warrants further investigation. On the basis of the initial limited number of identifications from the *in vitro* nitrated brain sample, we anticipate that there are still challenges for endogeneous nitrotyrosine profiling in tissue samples from various pathological conditions. Following further optimization or coupling with other immunoaffinity approaches and with the use of high sensitivity LC−MS/MS platform, the effectiveness of this approach may be fully illustrated for the analysis of an endogenous nitrotyrosine proteome sample.

Abbreviations: LC, liquid chromatography; HPLC, high-performance liquid chromatography; RPLC, reversed-phase liquid chromatography; UV, ultraviolet; MS, mass spectrometry; ECD, electrochemical detection; GC, gas chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SCX, strong cation exchange; CID, collision-induced dissociation; SPE, solid phase extraction; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline.

Acknowledgment. This work was supported in part by the Pacific Northwest National Laboratory LDRD program (W.-J.Q.), NIH grant AG018013 (D.J.B.), the National Center for Research Resources RR018522 (R.D.S.), and NIH grants R01 DA015802 and R01 NS050148 (D.J.S.). Much of the work was performed at the Environmental Molecular Science Laboratory, a national scientific user facility located at Pacific Northwest National Laboratory (PNNL) and sponsored by the U.S. Department of Energy (DOE) Office of Biological and Environmental Research. PNNL is operated by Battelle for the DOE under Contract No. DE-AC05-76RLO-1830.

Supporting Information Available: The list of non-nitrotyrosine-containing peptides identified from enriched sample of nitrated mouse brain tissue (Excel file). Product ion spectra for the sequencing of the original nitrotyrosine-containing standard peptide (PDF file). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Radi, R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (12), 4003−8.
(2) Gow, A. J.; Farkouh, C. R.; Munson, D. A.; Posencheg, M. A.; Ischiropoulos, H. Biological significance of nitric oxide-mediated protein modifications. *Am. J. Physiol.: Lung. Cell Mol. Physiol.* **2004**, *287* (2), L262−8.
(3) Dedon, P. C.; Tannenbaum, S. R. Reactive nitrogen species in the chemical biology of inflammation. *Arch. Biochem. Biophys.* **2004**, *423* (1), 12−22.
(4) Patel, R. P.; McAndrew, J.; Sellak, H.; White, C. R.; Jo, H.; Freeman, B. A.; Darley-Usmar, V. M. Biological aspects of reactive nitrogen species. *Biochim. Biophys. Acta* **1999**, *1411* (2−3), 385−400.
(5) Schopfer, F. J.; Baker, P. R. S.; Freeman, B. A. NO-dependent protein nitration: a cell signaling event or an oxidative inflammatory response? *Trends Biochem. Sci.* **2003**, *28* (12), 646−54.
(6) Sacksteder, C. A.; Qian, W. J.; Knyushko, T. V.; Wang, H.; Chin, M. H.; Lacan, G.; Melega, W. P.; Camp, D. G., II; Smith, R. D.; Smith, D. J.; Squier, T. C.; Bigelow, D. J. Endogenously nitrated proteins in mouse brain: links to neurodegenerative disease. *Biochemistry* **2006**, *45* (26), 8009−22.
(7) Aslan, M.; Ozben, T. Reactive oxygen and nitrogen species in Alzheimer's disease. *Curr. Alzheimer Res.* **2004**, *1* (2), 111−9.
(8) Butterfield, D. A. Proteomics: a new approach to investigate oxidative stress in Alzheimer's disease brain. *Brain Res.* **2004**, *1000* (1−2), 1−7.
(9) Flirski, M.; Sobow, T. Biochemical markers and risk factors of Alzheimer's disease. *Curr. Alzheimer Res.* **2005**, *2* (1), 47−64.
(10) Drew, B.; Leeuwenburgh, C. Aging and the role of reactive nitrogen species. *Ann. N. Y. Acad. Sci.* **2002**, *959*, 66−81.
(11) Schoneich, C. Mass spectrometry in aging research. *Mass Spectrom. Rev.* **2005**, *24* (5), 701−18.
(12) Schoneich, C. Protein modification in aging: An update. *Exp. Gerontol.* **2006**, *41*, 807−12.
(13) Sharov, V. S.; Galeva, N. A.; Kanski, J.; Williams, T. D.; Schoneich, C. Age-associated tyrosine nitration of rat skeletal muscle glycogen phosphorylase b: characterization by HPLC-nanoelectrospray-tandem mass spectrometry. *Exp. Gerontol.* **2006**, *41* (4), 407−16.
(14) Kanski, J.; Hong, S. J.; Schoneich, C. Proteomic analysis of protein nitration in aging skeletal muscle and identification of nitrotyrosine-containing sequences in vivo by nanoelectrospray ionization tandem mass spectrometry. *J. Biol. Chem.* **2005**, *280* (25), 24261−6.
(15) Kanski, J.; Schoneich, C. Protein nitration in biological aging: proteomic and tandem mass spectrometric characterization of nitrated sites. *Methods Enzymol.* **2005**, *396* 160−71.
(16) Ischiropoulos, H. Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem. Biophys. Res. Commun.* **2003**, *305* (3), 776−83.
(17) Koeck, T.; Fu, X.; Hazen, S. L.; Crabb, J. W.; Stuehr, D. J.; Aulak, K. S. Rapid and selective oxygen-regulated protein tyrosine denitration and nitration in mitochondria. *J. Biol. Chem.* **2004**, *279* (26), 27257−62.
(18) Beal, M. F. Oxidatively modified proteins in aging and disease. *Free Radical Biol. Med.* **2002**, *32* (9), 797−803.
(19) Crow, J. P. Measurement and significance of free and protein-bound 3-nitrotyrosine, 3-chlorotyrosine, and free 3-nitro-4-hydroxyphenylacetic acid in biologic samples: a high-performance liquid chromatography method using electrochemical detection. *Methods Enzymol.* **1999**, *301* 151−60.
(20) Herce-Pagliai, C.; Kotecha, S.; Shuker, D. E. Analytical methods for 3-nitrotyrosine as a marker of exposure to reactive nitrogen species: a review. *Nitric Oxide* **1998**, *2* (5), 324−36.
(21) Duncan, M. W. A review of approaches to the analysis of 3-nitrotyrosine. *Amino Acids* **2003**, *25* (3−4), 351−61.

(22) Tsikas, D.; Caidahl, K. Recent methodological advances in the mass spectrometric analysis of free and protein-associated 3-nitrotyrosine in human plasma. *J. Chromatogr.. B: Anal. Technol. Biomed. Life Sci.* **2005**, *814* (1), 1−9.
(23) Kanski, J.; Behring, A.; Pelling, J.; Schoneich, C. Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: effects of biological aging. *Am. J. Physiol.: Heart Circ. Physiol.* **2005**, *288* (1), H371−81.
(24) Kanski, J.; Alterman, M. A.; Schoneich, C. Proteomic identification of age-dependent protein nitration in rat skeletal muscle. *Free Radical Biol. Med.* **2003**, *35* (10), 1229−39.
(25) Sharov, V. S.; Galeva, N. A.; Knyushko, T. V.; Bigelow, D. J.; Williams, T. D.; Schoneich, C. Two-dimensional separation of the membrane protein sarcoplasmic reticulum Ca-ATPase for high-performance liquid chromatography-tandem mass spectrometry analysis of posttranslational protein modifications. *Anal. Biochem.* **2002**, *308* (2), 328−35.
(26) Petersson, A. S.; Steen, H.; Kalume, D. E.; Caidahl, K.; Roepstorff, P. Investigation of tyrosine nitration in proteins by mass spectrometry. *J. Mass Spectrom.* **2001**, *36* (6), 616−25.
(27) Ye, Y. Z.; Strong, M.; Huang, Z. Q.; Beckman, J. S. Antibodies that recognize nitrotyrosine. *Methods Enzymol.* **1996**, *269*, 201−9.
(28) Franze, T.; Weller, M. G.; Niessner, R.; Poschl, U. Comparison of nitrotyrosine antibodies and development of immunoassays for the detection of nitrated proteins. *Analyst* **2004**, *129* (7), 589−96.
(29) MacMillan-Crow, L. A.; Thompson, J. A. Immunoprecipitation of nitrotyrosine-containing proteins. *Methods Enzymol.* **1999**, *301*, 135−45.
(30) Nikov, G.; Bhat, V.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrated proteins by nitrotyrosine-specific affinity probes and mass spectrometry. *Anal. Biochem.* **2003**, *320* (2), 214−22.
(31) Liu, T.; Qian, W. J.; Strittmatter, E. F.; Camp, D. G., II; Anderson, G. A.; Thrall, B. D.; Smith, R. D. High-throughput comparative proteome analysis using a quantitative cysteinyl-peptide enrichment technology. *Anal. Chem.* **2004**, *76* (18), 5345−53.
(32) Knyushko, T. V.; Sharov, V. S.; Williams, T. D.; Schoneich, C.; Bigelow, D. J. 3-Nitrotyrosine modification of SERCA2a in the aging heart: a distinct signature of the cellular redox environment. *Biochemistry* **2005**, *44* (39), 13071−81.
(33) Gevaert, K.; Goethals, M.; Martens, L.; Van, Damme, J.; Staes, A.; Thomas, G. R.; Vandekerckhove, J. Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides. *Nat. Biotechnol.* **2003**, *21* (5), 566−9.
(34) Sokolovsky, M.; Riordan, J. F.; Vallee, B. L. Conversion of 3-nitrotyrosine to 3-aminotyrosine in peptides and proteins. *Biochem. Biophys. Res. Commun.* **1967**, *27* (1), 20−5.
(35) Thannhauser, T. W.; Konishi, Y.; Scheraga, H. A. Sensitive quantitative analysis of disulfide bonds in polypeptides and proteins. *Anal. Biochem.* **1984**, *138* (1), 181−8.
(36) Shen, Y.; Tolic, N.; Zhao, R.; Pasa-Tolic, L.; Li, L.; Berger, S. J.; Harkewicz, R.; Anderson, G. A.; Belov, M. E.; Smith, R. D. High-throughput proteomics using high-efficiency multiple-capillary liquid chromatography with on-line high-performance ESI FTICR mass spectrometry. *Anal. Chem.* **2001**, *73* (13), 3011−21.
(37) Qian, W. J.; Liu, T.; Monroe, M. E.; Strittmatter, E. F.; Jacobs, J. M.; Kangas, L. J.; Petritis, K.; Camp, D. G., II; Smith, R. D. Probability-based evaluation of peptide and protein identifications from tandem mass spectrometry and SEQUEST analysis: the human proteome. *J. Proteome Res.* **2005**, *4* (1), 53−62.
(38) Wang, H.; Qian, W. J.; Chin, M. H.; Petyuk, V. A.; Barry, R. C.; Liu, T.; Gritsenko, M. A.; Mottaz, H. M.; Moore, R. J.; Camp, Ii, D. G.; Khan, A. H.; Smith, D. J.; Smith, R. D. Characterization of the mouse brain proteome using global proteomic analysis complemented with cysteinyl-peptide enrichment. *J. Proteome Res.* **2006**, *5* (2), 361−9.
(39) Duncan, R. J.; Weston, P. D.; Wrigglesworth, R. A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. *Anal. Biochem.* **1983**, *132* (1), 68−73.
(40) Smallwood, H. S.; Galeva, N. A.; Bartlett, R. K.; Urbauer, R. J.; Williams, T. D.; Urbauer, J. L.; Squier, T. C. Selective nitration of Tyr99 in calmodulin as a marker of cellular conditions of oxidative stress. *Chem. Res. Toxicol.* **2003**, *16* (1), 95−102.
(41) Ghesquiere, B.; Goethals, M.; Van Damme, J.; Staes, A.; Timmerman, E.; Vandekerckhove, J.; Gevaert, K. Improved tandem mass spectrometric characterization of 3-nitrotyrosine sites in peptides. *Rapid Commun. Mass Spectrom.* **2006**, *20* (19), 2885−93.
(42) Yamakura, F.; Taka, H.; Fujimura, T.; Murayama, K. Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J. Biol. Chem.* **1998**, *273* (23), 14085−9.
(43) Viner, R. I.; Ferrington, D. A.; Williams, T. D.; Bigelow, D. J.; Schoneich, C. Protein modification during biological aging: selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca2+-ATPase in skeletal muscle. *Biochem. J.* **1999**, 340 (Pt 3), 657−69.
(44) Heller, M.; Mattou, H.; Menzel, C.; Yao, X. Trypsin catalyzed 16O-to-18O exchange for comparative proteomics: tandem mass spectrometry comparison using MALDI-TOF, ESI-QTOF, and ESI-ion trap mass spectrometers. *J. Am. Soc. Mass Spectrom.* **2003**, *14* (7), 704−18.
(45) Qian, W. J.; Monroe, M. E.; Liu, T.; Jacobs, J. M.; Anderson, G. A.; Shen, Y.; Moore, R. J.; Anderson, D. J.; Zhang, R.; Calvano, S. E.; Lowry, S. F.; Xiao, W.; Moldawer, L. L.; Davis, R. W.; Tompkins, R. G.; Camp, D. G., II; Smith, R. D. Quantitative proteome analysis of human plasma following in vivo lipopolysaccharide administration using 16O/18O labeling and the accurate mass and time tag approach. *Mol. Cell. Proteomics* **2005**, *4* (5), 700−9.

PR0606934