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CRITICAL INSIGHT

When is Mass Spectrometry Combined with Affinity Approaches Essential? A Case Study of Tyrosine Nitration in Proteins

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Abstract

Tyrosine nitration in proteins occurs under physiologic conditions and is increased at disease conditions associated with oxidative stress, such as inflammation and Alzheimer's disease. Identification and quantification of tyrosine-nitrations are crucial for understanding nitration mechanism(s) and their functional consequences. Mass spectrometry (MS) is best suited to identify nitration sites, but is hampered by low stabilities and modification levels and possible structural changes induced by nitration. In this insight, we discuss methods for identifying and quantifying nitration sites by proteolytic affinity extraction using nitrotyrosine (NT)-specific antibodies, in combination with electrospray-MS. The efficiency of this approach is illustrated by identification of specific nitration sites in two proteins in eosinophil granules from several biological samples, eosinophil-cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). Affinity extraction combined with Edman sequencing enabled the quantification of nitration levels, which were found to be 8 % and 15 % for ECP and EDN, respectively. Structure modeling utilizing available crystal structures and affinity studies using synthetic NT-peptides suggest a tyrosine nitration sequence motif comprising positively charged residues in the vicinity of the NT- residue, located at specific surface- accessible sites of the protein structure. Affinities of Tyr-nitrated peptides from ECP and EDN to NT-antibodies, determined by online bioaffinity- MS, provided nanomolar K_D values. In contrast, false-positive identifications of nitrations were obtained in proteins from cystic fibrosis patients upon using NT-specific antibodies, and were shown to be hydroxy-tyrosine modifications. These results demonstrate affinity- mass spectrometry approaches to be essential for unequivocal identification of biological tyrosine nitrations.

Key words: Affinity-mass spectrometry combination, Protein tyrosine nitration, Nitro-tyrosine antibodies, Eosinophil proteins, Proteolytic affinity extraction-mass spectrometry, Quantification of nitrations, False-positive identification

Introduction

Tyrosine nitration represents a prominent pathway of protein oxidative modification that occurs under physiologic conditions. It is found in an increasing number of

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pathophysiologic conditions such as atherosclerosis, asthma and lung diseases, neurodegenerative diseases, and diabetes [1–9]. Nitration only occurs on specific proteins and on specific tyrosine residues. This specificity may be regulated by intracellular localization, structure and folding of proteins, and concentrations and reactivities of nitrating species [10, 11]. Nitration of proteins has been mainly identified in vitro in studies with known proteins, the physiologic relevance of which remains to be determined. A number of analytical methods for detecting and identifying nitrations have been employed with a broad range of specificities and sensitivities, and with the risk of comprising both false positive and false negative identifications [12–14]. Most identifications of nitrations have been derived from the use of anti-3-nitrotyrosine (3-NT) antibodies; however, only scarce information of properties and molecular specificities of 3-NT-antibodies has been hitherto reported [15]. Immunoanalytical methods such as Western blot [16, 17], ELISA [18–20] and immuno-electron microscopy [20], employing different 3-NT-antibodies, have been used for overall detection of nitrations, but do not provide identification of nitration sites and structures.

The identification and structural characterization of tyrosine nitrations is a challenging task for mass spectrometry. Previous studies have shown that the mass spectrometric identification of nitration sites may be hampered by (1) low levels of nitration in biological samples; (2) instability of reactive nitrating agents; and (3) instability of nitrated proteins at the condition of MS analysis [21–23]. Thus, UV-MALDI-MS has been found to be critical for unequivocal identification of nitrations from biological samples due to the photochemical decomposition of nitrotyrosine residues [24, 25]. In contrast, electrospray ionization mass spectrometry and IR-MALDI-MS have been shown to provide unambiguous identifications of NT-modified peptides [25–27]. In previous studies, different approaches of MS have been successfully employed for identifying tyrosine nitrations upon reaction with peroxynitrite in vitro [12, 28] and in vivo [3]. However, since nitration in vivo may occur at low and varying concentrations of proteins and with only low extent of modification, detection, and structure determination by conventional analytical approaches may present considerable difficulties, thus requiring methods for specific enrichment of nitrated proteins prior to MS analysis.

Recent work in our laboratory has focused on the development of combined affinity- mass spectrometry methods for the identification of biomolecular recognition structures, such as protein antigen-epitopes [29–33]. In this CI we describe a general approach for the structural identification of protein nitrations by a combination of proteolytic affinity extraction and ESI mass spectrometry (Figure 1). This approach is illustrated by the identification of the specific nitration sites in vivo of human eosinophil proteins, and characterization of their structural environment. Moreover, the affinity-MS approach employed provides a basis for the quantitative determination of the extent

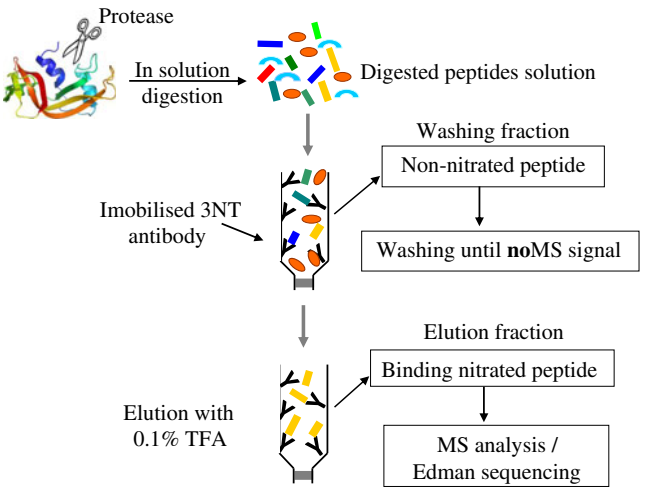


Figure 1. Principle of proteolytic affinity extraction-MS using an immobilized 3-NT antibody. Peptides or proteins (protein complexes) are digested in solution and the resulting peptide mixtures presented to the sepharose-immobilized antibody column. Washing steps are performed to remove nonbinding peptide fragments; the remaining affinity-bound peptide(s) are eluted and analyzed by mass spectrometry. The mouse monoclonal anti-3-NT antibody (MAB5404; Chemicon International, Bubendorf, Switzerland), was produced using nitrated KLH as an immunogen

of nitrations using Edman sequencing of affinity-extracted proteolytic peptides, and for the molecular assessment of site selectivity and topology of tyrosine nitrations. In contrast to these studies of physiologic nitrations, we present examples in a study of oxidative modifications in proteins from cystic fibrosis patients that false-positive assignment of nitrations may result from employing only NT-specific antibodies without using mass spectrometry, thus illustrating that the combination of affinity approaches and mass spectrometry is essential for unequivocal identification of tyrosine nitration in proteins.

Proteolytic Affinity-Mass Spectrometry Approach for Identification of Tyrosine Nitration Sites

A 3-NT antibody-affinity column was prepared and employed for the proteolytic affinity extraction-MS approach for identification of nitration sites, as shown in Figure 1. The principle of this approach is analogous to a general, previously developed epitope extraction-MS method for identification of antibody-epitopes, in which a protein antigen is digested in solution and the proteolytic peptide mixture presented to the affinity column [32, 33]. The 3-NT-antibody–peptide(s) complex is allowed to form, and nonbinding peptides remaining in solution are removed by washing and analyzed by mass spectrometry as a supernatant fraction. According to the antibody's specificity, only nitrated peptides remain bound to the 3-NT-affinity column for subsequent mass spectrometric analysis. The affinity-matrix is washed extensively to ensure complete removal of

unbound peptides. Peptides remaining bound to the antibody are then eluted at slightly acidic conditions (pH 2–3; elution fraction). Importantly, the specificity of the 3NT-antibody is tested and ascertained independently by affinity-MS using synthetic tyrosine-nitrated model peptides [3].

The feasibility and efficiency of this approach was first shown by the identification of tyrosine nitration sites in two eosinophil proteins purified from eosinophil granules (a kind gift of Dr. H. Kita, Mayo Clinic, Rochester) of patients with cystic fibrosis, eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) (Figures 2 and 3; Table 1) [34, 35]. Eosinophil proteins were separated by 1D-gel electrophoresis under denaturing conditions, transferred onto a nitrocellulose membrane, and stained with anti-3NT antibody (Figure 2). Due to their high sequence homology, EDN and ECP are observed at similar molecular masses (~16 kDa; bands 1, 3 in Figure 2a); additional bands at higher masses (~20 and 23 kDa; bands 2, 4) correspond to glycosylated forms, since both eosinophil proteins contain N-glycosylation sites [36, 37]. The Western blot analyses showed that the glycosylated forms are nitrated, indicating that glycosylation is within the domain of the nitrated tyrosine residues. Bands 1 and 3 were excised, digested with trypsin and the resulting peptide fragments first analyzed by MALDI-TOF-MS. MALDI-MS of the peptide fragments provided identification for both proteins; however, no nitrated peptides and nitration sites were found (Figures S1, S2; Supporting Information). These results and studies of other nitrated proteins from biological samples clearly indicate the low extent of modification as a major problem for the identification of nitration sites, in the presence of an excess of unmodified protein (see also below).

Unequivocal identifications of tyrosine nitrations were obtained by proteolytic-affinity extraction–MS (Figure 3 for EDN). EDN was cysteine-carbamoylated and then digested in solution with thermolysin, used instead of trypsin due to the low number of Arg and Lys residues in the N-terminal part of the protein. The mixture of proteolytic peptides produced was submitted to the affinity column, incubated for 2 h, and the supernatant non-binding peptides removed by washing and analyzed by MALDI-TOF-MS as a control (Figure S3, Supporting Information). The remaining antibody-peptide complex was then dissociated at slightly acidic conditions, and the elution fraction analyzed by high resolution FTICR- mass spectrometry. The ESI-FTICR-MS revealed a single Tyr-peptide, EDN (^{29}VINNY(NO_2)QRRCKNQNTF^{43}) (Figure 3) with a monoisotopic mass corresponding to a single nitration at Tyr^{33} with a mass increment of 103 Da (45 Da for the Tyr^{33}-nitro group, 58 Da for Cys^{37}-carbamidomethyl; 7 ppm mass accuracy). For the affinity-isolated peptide, confirmation of the nitration site was readily obtained by tandem-MS sequencing using infrared multiphoton dissociation (IRMPD) [36] (Figure S4, Supporting Information).

In an analogous manner, application of the proteolytic affinity-MS approach to ECP provided the identification of a single nitration site in the proteolytic peptide obtained by trypsin digestion, ECP(^{23}CTIAMRAINNY(NO_2)R^{34}) nitrated at Tyr^{33}. The uncleaved Arg^{28} residue suggested that the proteolytic resistance at this residue near the Tyr^{33} residue might be due to the nitration, since in non- nitrated ECP the same arginine residue was cleaved by trypsin; the observation that proteolytic cleavage is affected by a nearby Tyr-nitration site has been made in several studies on protein

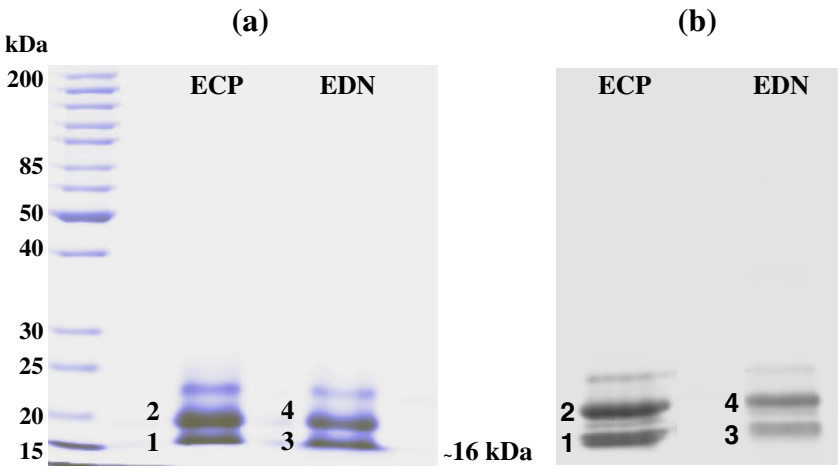


Figure 2. SDS gel electrophoresis of ECP and EDN from electrophoretic separation of human eosinophil granules, visualized by colloidal Coomassie **(a)** and Western blot using a monoclonal anti-3-nitro-tyrosine antibody (MAB5404) **(b)**. Bands 1, 2 correspond to ECP, bands 3, 4 to EDN, each protein showing several glycosylations. Eosinophil proteins were isolated from four patients with hypereosinophilia and elevated eosinophils, using a previously described protocol [36]. All samples were obtained according to approved protocols by the Ethics Committee of Mayo Clinic Laboratories (Rochester, USA) and upon informed consent of patients. Pure eosinophil samples were obtained from the Institute of Medical Microbiology and Hygiene, Universitätsklinikum Tübingen. Sputum samples were extracted from a 28-year-old cystic fibrosis patient chronically infected with *P. aeruginosa*, and were also provided by our collaborators of the Universitätsklinikum Tübingen

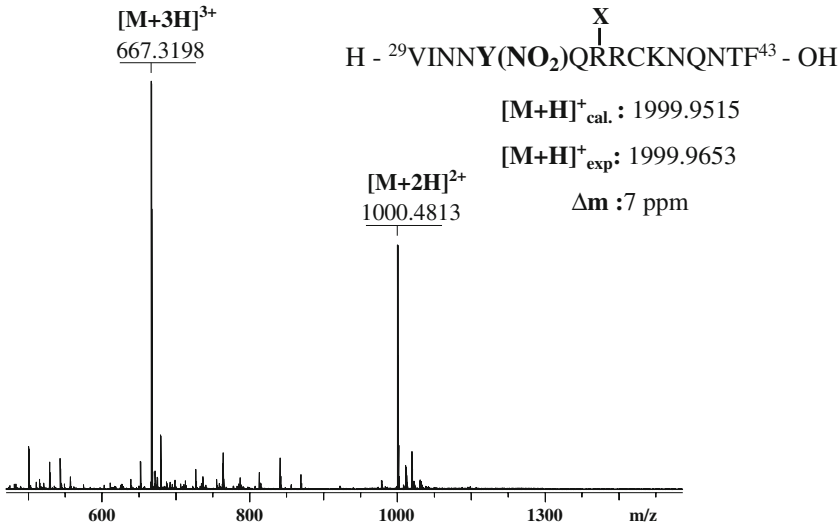


Figure 3. Identification of the tyrosine nitration site at Tyr –33 in eosinophil-derived neurotoxin by nano-ESI-FTICR-MS of the thermolysine peptide fragmnent, EDN (29–43). The modification denoted with X corresponds to carbamidomethylation at Cys-37. Prior to the digestion of eosinophil protein solutions, reduction of disulfide bonds was performed in two steps; (i), proteins were first dissolved to a concentration of 1 μg/μL in 10 mM NH_4HCO_3 (pH 8); (ii), DTT in 10 mM NH_4HCO_3 was added at a 50-fold excess relative to the number of disulfide bonds, and reduction was carried out for 1 h at 56 °C under gentle shaking. After cooling to 20 °C an aqueous solution of iodoacetamide was added at a 2.2- old excess and the reaction performed for 45 min at 20 °C in the dark. Following lyophilization, ECP protein was digested with trypsin in 10 mM NH_4HCO_3, pH 8. The enzyme to substrate ratio was 1:30 (wt/wt), and the digestion was carried out for 4 h at 37 °C. For eosinophil derived-neurotoxin digestion was performed for 6 h at 60 °C a ratio of 1:50 (wt/wt) thermolysin: protein in 50 mM Tris, pH 8.0, containing 5 mM $CaCl_2$ and 10 % (vol/vol) acetonitrile. Both reactions were quenched by freezing with liquid nitrogen; proteolytic peptide mixtures were analyzed directly by mass spectrometry, or used for immunoaffinity experiments. Proteolytic affinity- extraction of peptides was performed with an anti-3NT antibody column prepared as previously described [3, 36] using 100 μg of the MAB5404 antibody. Following the final washing step, the column was kept in 10 mL PBS buffer and stored at 4 °C. Proteolytic peptide fragments were added onto the antibody column and incubated for 2 h at 20 °C under gentle shaking. Unbound peptides (supernatant fraction) were removed by blowing out the column to near-complete dryness using a 10 mL syringe, and the matrix was then washed with 200 mL PBS buffer. The nitrated peptide–antibody complex was then dissociated by addition of 0.1 % TFA, and the elution fractions analyzed by MALDI-TOF-MS and ESI- FTICR-MS

nitrations [28, 36]. For the ECP and EDN proteins, the identification of analogous specific Tyr- nitration sites is in agreement with the high degree of structural homology for both proteins [37].

These results demonstrate specific tyrosine nitrations in Eosinophil proteins and illustrate the proteolytic affinity-extraction- MS as a highly efficient approach, far more specific and sensitive compared with LC-MS/MS approaches employed in previous studies [26, 27] and in the identification of the self-nitration of eosinophil peroxidase (EPO) [38]. Moreover, the proteolytic affinity-extraction- MS of producing and submitting a peptide mixture is applicable to single proteins, protein complexes from biological material and protein bands from electrophoretic separations with comparable efficiency [28, 36]. Other approaches using affinity-enrichment in conjunction with tandem-MS have successfully yielded unequivocal identifications of tyrosine-nitrations [39, 40]. In contrast, the direct identification of nitrations without affinity- extraction using HPLC-MS, such as in the auto-nitration site of eosinophil-peroxidase (EPO) at Tyr^{349} required highly purified protein and was only obtained after scrutinous HPLC separation of

Table 1. Quantitative Determination of Nitro-Tyrosine Modifications in Eosinophil Proteins by Edman Sequencing of Nitrated Peptides

Eosinophil protein	(10^{-9} mol) protein[a]	Nitrated peptide[b]	(10^{-9} mol) nitrated peptide[c]	Amount of nitrated peptide[d]
ECP	12.25	CTIAMRAINN**Y(NO$_2$)**R	2.10	15.9
EDN	13	VINN**Y(NO$_2$)**QRRCKNQNTF	1.27	9.2

[a] Total amount of protein used for affinity – Edman sequencing approach.
[b] Nitrated peptide sequence identified by affinity – Edman sequencing approach.
[c] Amount of nitrated tyrosine peptides determined using the initial yield by Edman sequencing.
[d] Percent of nitrated tyrosine peptides contained in eosinophil proteins determined using the repetitive yield of Edman sequencing. The repetitive yield was 92.7 for ECP peptide and 93.2 for EDN peptide, respectively.

Table 2. Sequences in the Vicinity of Nitration Sites Identified for Eosinophil Proteins and Prostacyclin Synthase

Protein	Sequence motif -X**BB**X**B**Z**n**Y**B**X**B**X**B**-[a]
Eosinophil cationic protein	^{23}CTIAM**R**AINN**nYR**^{34}
Eosinophil-derived neurotoxin	^{29}VINN**nY**Q**RR**C**K**NQNTF^{43}
Eosinophil peroxidase	^{333}FGHTMLQPFMF**R**LDSQ**nYR**^{350}
Prostacyclin synthase	^{424}G**KR**L**K**N**n**Y**S**^{431}

[a] **X**=any amino acid; **B**=basic/ K, R; Z=neutral; **nY**=nitrated tyrosine.

the tryptic peptide, EPO(^{333}FGHTMLQPFMFRLDSQ**Y(NO$_2$)**R^{350}) [3, 36] (s. Table 2). Like in ECP, an uncleaved residue, Arg^{344} was noted near the Tyr-nitration site. The high specificity of in vivo nitration in proteins was ascertained in eosinophil granules in human and animal tissue, and in leukocytes of cystic fibrosis patients, and tyrosine nitration was shown to be specifically catalyzed by eosinophil peroxidase EPO [3].

A large scale study using tandem-MS has been reported by Prokai et al. who described factors that may provide false-positive identifications of protein nitrations [40, 41] and emphasized the importance of manually validating nitration sites by using defined synthetic peptides. In a study of the nitration in ß-actin in sickle cell disease, a related approach to proteolytic affinity-extraction has been employed by Aslan et al. [42], who used affinity-isolation of the complete protein, followed by in-gel digestion and mass spectrometric analysis using both MALDI- and ESI-MS, which resulted in identifications of multiple nitration sites. Several nitrations were found in vitro with peroxynitrite, while nitrotyrosine modifications were not detected in vivo, suggesting that nitration sites might be shielded in cellular protein complexes. In this study, intact protein bands from gel electrophoresis successfully yielded identifications of Tyr-nitrations. However, due to the significant problems encountered in affinity-isolations of intact proteins and protein complexes [28, 36], the affinity-MS method using proteolytic peptide mixtures appears to be a more facile, generally applicable approach for identification of nitration sites. In contrast, direct "top-down" molecular weight determination of a nitrated protein would require high-accuracy mass spectrometric methods such as FTICR-MS, and does not seem to be a generally feasible approach because of the typically low extent of nitrations. Independent molecular weight characterization of a nitrated protein can be readily obtained by gel electrophoresis and Western Blot (Figure 2 for eosinophil proteins).

Quantitative Determination of Protein Nitration Levels

The proteolytic affinity- MS approach enables the identification of specific nitration sites in physiological proteins, but provides no direct quantitative information of the extent of nitration. In previous studies, no quantitative data of the extent of Tyr-nitrations have been reported. An Edman sequencing approach of affinity-extracted nitrated peptides was developed for quantitative determination of tyrosine nitration levels, and was successfully applied to eosinophil proteins [36]. Proteolytic affinity extraction was performed for both ECP and EDN proteins, and quantification obtained by determination of initial and repetitive yields in each sequencing cycle of the amino acids including the nitrated tyrosine residues (Figure S5, S6; Supporting Information). Quantitative determinations from the ratios of nitrated peptides to intact proteins provided approximately 15 % and 8 % nitration levels for ECP and EDN, respectively (Table 1). These results were consistent with Western blot data that indicated a higher detection sensitivity for ECP compared to EDN.

It should be noted that quantitative determinations of the relative extent of nitrations using repetitive Edman sequencing can be carried out both with nitrated proteins in vitro,

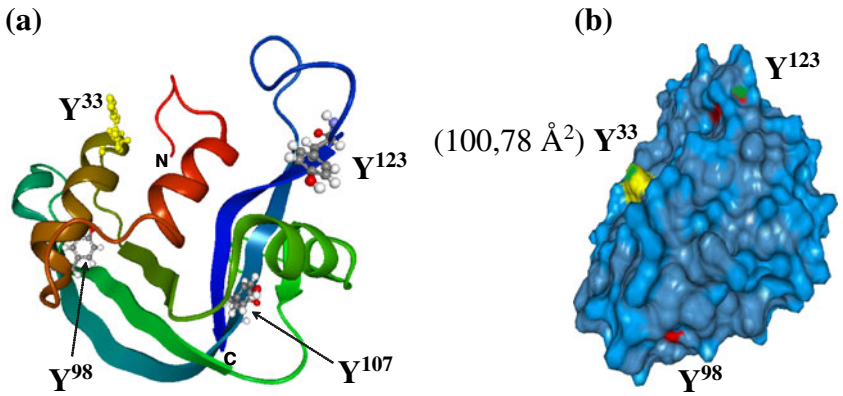
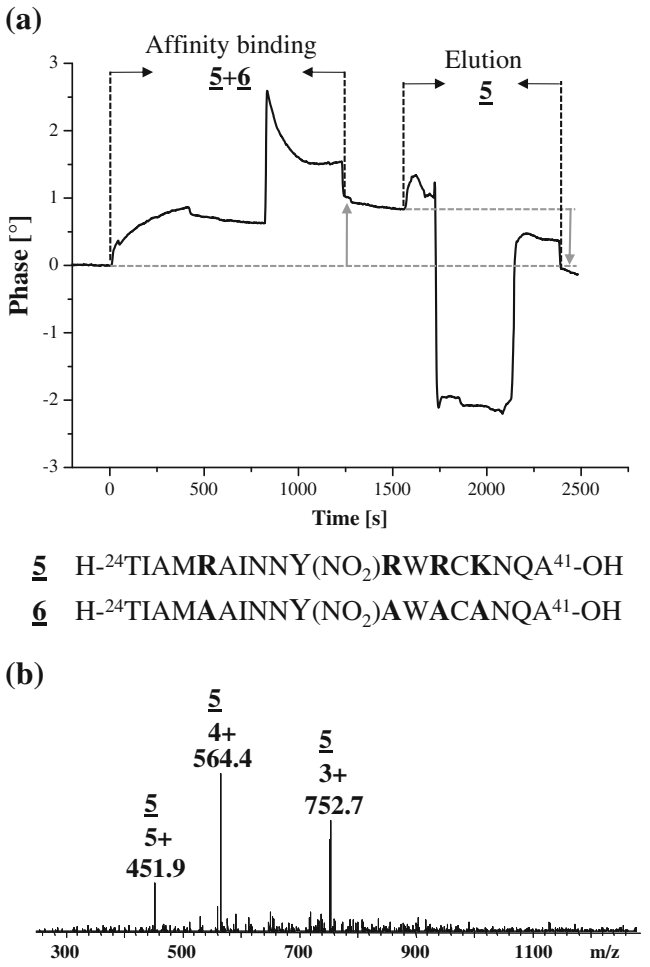


Figure 4. Molecular modeling and structure of eosinophil-derived neurotoxin. **(a)** Ribbon diagram indicating the location of all four tyrosine residues of EDN (nitrated Tyr^{33} is colored in yellow) and **(b)** solvent surface accessibility (SAS) of EDN. The locations of surface exposed tyrosine residues are indicated by colors as follows: yellow for Tyr^{33}, red for Tyr^{98}, and Tyr^{123} and green for tyrosine hydroxyl groups. The structure modeling was performed using BallView 1.1.1 program based on the available X-ray crystal structure of EDN (PDB entry 1GQV)



◀ **Figure 5.** Biosensor curves of ECP peptide mixture (5+6) using online bioaffinity-MS **(a)** and ESI-MS identification of the Tyr-nitrated peptide 5 bound to the anti-3-nitrotyrosine antibody (MAB5404) **(b)**. *N*-α-Fmoc-3-nitrotyrosine, *N*-α-Fmoc-protected amino acids, and PyBOP activator for peptide synthesis were obtained from Bachem (Bubendorf, Switzerland). *N*-α-Fmoc protected amino acids attached to NovaSyn TGA resin was from NovaBiochem (Laufelfingen, Switzerland). A K5-Ssens Biosensor (SAW-Instruments, Bonn, Germany) was used for online bioaffinity-MS with an interface developed in our laboratory, as previously described [50]. The antibody was immobilized on a gold chip, following formation of a self-assembled monolayer (SAM) surface of 16- mercaptohexadecanoic acid for 12 h at 25 °C. SAM-carboxy groups were activated with 200 mM *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC), and coupling performed with 50 mM NHS followed by washing of the chip surface with 40 µL min^{-1} H_2O. A solution of 300 nM (150 µL) antibody was immobilized on the SAM, the remaining NHS groups blocked with 1 M ethanolamine, and a 10 µM peptide solution then added, followed by affinity binding at a flow rate of 20 µL min^{-1}. Following ligand association, elution was carried out with glycine buffer, and removal of salts was performed by washing with 0.3 % HCOOH. Elution and transfer into the ESI source of a Bruker Esquire 3000+ mass spectrometer was performed as previously described [50]. Determination of dissociation constants was performed with 150 µL (300 nM) immobilized antibody at a 20 µL min^{-1} flow rate in PBS buffer, pH 7.5, followed by regeneration with 150 µL glycine buffer, pH 2. Changes of sensor-phase signals were fitted with the programs OriginPro 7.5 (OriginLab Corp., Northampton, MA, USA) and FitMaster (SAW-Instruments, Bonn, Germany). Determination of antibody/protein association kinetics was performed by extracting the data from the sensor signals using the monomolecular growth model [47]. Determinations of dissociation constants was performed by plotting the pseudo first-order kinetic constants for two independent channels verss concentration, and applying linear regression for $K_D = k_{off}\, k_{on}^{-1}$

and with cellular proteins [36]. However, this approach generally requires the chromatographic isolation of either the modified peptide or the intact nitrated protein, depending on the site of nitration within the polypeptide sequence.

Site Selectivity of Tyrosine Nitration in Proteins

Based on the available crystal structures of ECP and EDN [43, 44], the topologies and orientation of the identified nitrated tyrosine structures were assessed using the BallView molecular modeling program (Figure 4). Accessible surface areas were calculated for both proteins using the Surface Racer program [45]. The spatial orientation was compared for all tyrosine residues in EDN (PBD accession number 1GQV) and showed that only Tyr^{33} is located within a helical conformation (Figure 4a). The solvent accessible surface structure of EDN clearly showed the Tyr^{33} residue having the highest surface accessibility (100.8 $Å^2$), compared with other tyrosine residues, Tyr^{123} (60.5 $Å^2$) and Tyr^{98} (23.0 $Å^2$), while Try^{107} is completely embedded in the protein structure. The ECP protein revealed an analogous surface structure with Tyr^{33} having the highest accessibility (94.4 $Å^2$), while other Tyr residues are embedded in the inner protein structure.

These results suggest that the selectivity of nitration may depend on both suitable surface accessibilities for nitration in a NT-modified protein structure and a specific sequence in the vicinity of a nitration site. In order to characterize the possible effect of the sequence environment of an NT-residue, the affinities and specificities of the monoclonal anti-3-NT antibody (legend to Figure 1) used for the proteolytic affinity extraction-MS analysis were assessed by an indirect ELISA method [33], using synthetic Tyr-nitrated peptides comprising all tyrosine residues in ECP (Table S1, Figure S7; Supporting Information). The Tyr^{33}-nitrated peptide ECP(28–38) showed substantially higher affinity compared with all other nitrated ECP peptides; in contrast, the Tyr^{98}-nitrated peptide which is completely buried in the protein structure, revealed only minimal affinity. These results showed that the affinity differences between the nitrated ECP peptides correlate with a specific sequence environment of the tyrosine residues. Moreover, they are consistent with a recently described sequence motif

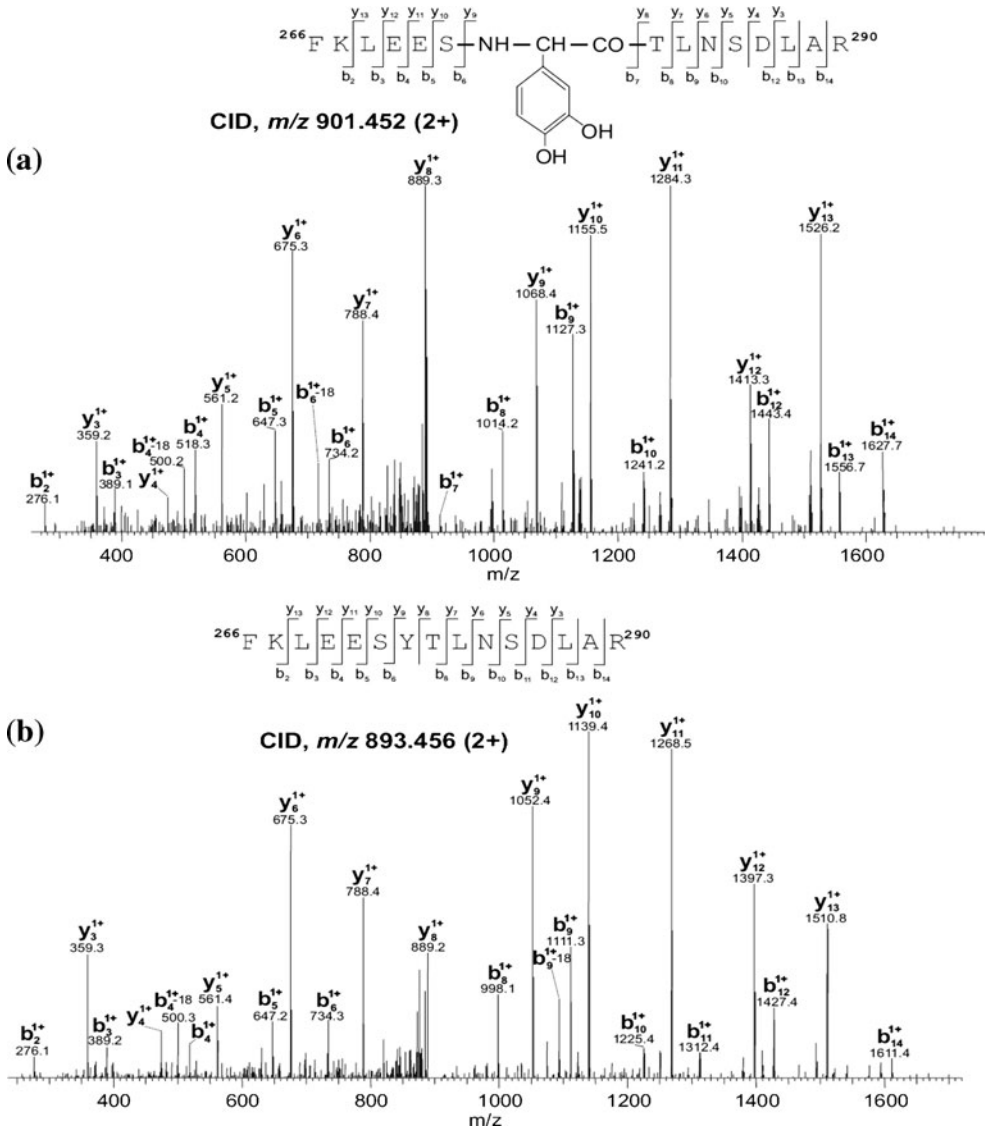


Figure 6. Nano-HPLC-tandem MS analysis of leukocyte elastase inhibitor revealed the identification of hydroxy-tyrosine modification. The sputum protein mixture of a patient with cystic fibrosis was separated by 2D-gel electrophoresis, and the corresponding protein spot digested with trypsin followed by MS analysis of the peptide fragments. **(a)** CID of the precursor ion *m/z* 901.452 (2+) of the tryptic peptide (266–290) showing hydroxy-tyrosine modification of Tyr-272; **(b)** precursor ion *m/z* 893.456 (2+) of the unmodified peptide (266–290)

for nitration sites, comprising cationic amino acids in the vicinity of nitrated tyrosine residues (Table 2) [46]. The possible role of sequence and structural environment for nitration has been discussed by several authors [11, 23, 33, 36, 40, 46–48]. Ischiropoulos et al. [47] have pointed out the importance of the protein structure for regulation of nitration sites, which is consistent with our results on surface accessibilities in the nitration of eosinophil proteins (Figure 4). A specific sequence prerequisite for nitration, analogous to the specific sequence for tyrosine phosphorylation by tyrosine kinases [49], has not yet been established from the present studies, and more detailed work employing tyrosine-nitrated model peptides is required to obtain more conclusive results. Thus, the present studies suggest that the nitration sequence environment, particularly charged amino acids, the accessible surface area within the protein structure, and the flexibility of domains comprising nitration sites, may be controlling factors

Table 3. Mass Spectrometric Determination of Hydroxy-Tyrosine Modification Sites of Leukocyte Elastase Inhibitor and Lactotransferrin, Using Proteolytic Affinity- Extraction with the Anti- 3NT Antibody

Protein	Peptide Sequence -X**BB**X**B**Z*Y **BXBXB**-[a]
Leukocyte elastase inhibitor	^{204}FAGYG*Y(209)IEDLK^{213}
	^{266}FKLEES*Y(272)TLNSDLAR^{290}
Lactotransferrin	^{333}IDSGLYLGSG*Y(343)FTAIQNLR^{351}
	^{406}GEADAMSLDGDGG*Y(419)VYTAGK^{423}
	^{681}YLGPQ*Y(686)VAGITNLK^{694}

[a] **X** = any amino acid; **B** = basic/ K, R; Z = neutral; ***Y** = hydroxy-tyrosine.

for tyrosine nitration sites. Moreover, these factors may be different for different mechanisms of nitration, such as nitration by peroxynitrite and peroxidase-catalyzed nitration in vivo.

A direct online combination of bioaffinity and mass spectrometry [50], a new method recently developed in our laboratory was employed for the simultaneous characterization and quantification of the interaction of tyrosine-nitrated peptides with the anti-3NT-antibody. The online affinity-MS approach utilized a surface-acoustic wave (SAW) biosensor [51], with the anti-3NT antibody immobilized on the biosensor chip surface via a self-assembled monolayer. Following association of peptides, dissociation was performed using an acidic glycine buffer into the interface for ESI-MS analysis [46]. Biosensor association curves and subsequent dissociation of affinity-bound ECP peptides from a mixture of nitrated and non-nitrated peptides are shown in Figure 5a. The ESI mass spectra of the affinity-eluate showed exclusively ions of the nitrated ECP peptide (Figure 5b). The affinity of the nitrated ECP peptide was found to be completely abolished upon replacing the positively charged residues Arg^{28}, Arg^{34}, Arg^{36}, and Lys^{38} by alanine, thus confirming the importance of cationic amino acids in the vicinity to the nitration site for affinity binding. Affinity determinations of the nitrated peptide in comparison to the intact ECP protein using the SAW biosensor provided dissociation constants of approximately 6 nM for the nitrated peptide ECP (24–41), and 28 nM for the intact ECP.

Hydroxy-Tyrosine Modification May Occur as False-Positive "Nitration" in Pathophysiologic Protein Modifications

In a proteomics study of sputum proteins from patients with cystic fibrosis, the proteolytic affinity extraction-MS approach was applied in order to detect and identify presumed protein nitrations using the identical anti-3NT antibody employed for the analysis of nitrated eosinophil proteins [3]. Sputum proteins were extracted from a 28-y-old cystic fibrosis patient chronically infected with *P. aeruginosa*, and were separated by 2D-gel electrophoresis, which had been previously established for the characterization of constituent proteins from complex biological samples, such as protein expression in bronchoalveolar lavage fluid [52, 53].

The isolated sputum proteins (see description in the legend to Figure 6) were suspended in lysis buffer and after concentration precipitated and applied to 1D- and 2D-gel electrophoretic separation and to Western blot analysis on a PVDF membrane, using the anti-3-NT antibody. Several positively staining, presumably nitrated proteins were detected on a 2D-proteome map. Among these, the leucocyte elastase inhibitor was of particular interest since this major serine protease inhibitor is usually found in human plasma and in lower respiratory tract regulating the fragmentation of elastin, fibronectin, and cell receptors on neutrophils [54]. Mass spectrometric identifications of Tyr-modifications upon proteolytic affinity extraction from two proteins, leucocyte elastase inhibitor and lactotransferrin, are shown in Figure 6 and Table 3. Leucocyte elastase inhibitor contains 10 tyrosine residues, all of which were identified within the proteolytic peptides. All Tyr-peptides were manually verified in order to identify nitrations and/or other possible modifications. Among the tyrosine residues, two (Tyr^{272} and Tyr^{209}) were found to be oxidized to hydroxy-tyrosine, and these modifications were confirmed by tandem-MS sequence determinations (Figure 6; Table 3). In addition, Lactotransferrin was found to be modified by hydroxylation at 3 tyrosine residues (Table 3).

Conclusions

The structural identification and quantification of nitrotyrosine modifications in proteins present particular challenges because of the low levels of modifications in vivo and the high potential for false positive and artefact results, both requiring analytical methods of high molecular specificity and sensitivity. The combination of proteolytic affinity extraction and ESI mass spectrometry is shown here as a highly efficient tool that is generally applicable to the molecular identification of nitration sites in biological proteins and protein complexes. Since identification is performed at the peptide level, this enables a reliable assignment of nitration sites. Moreover, Edman sequencing of the affinity-extracted nitrated peptides represents a sensitive approach for quantitative determination of the extent of nitrotyrosine modifications, and molecular modeling and biosensor-affinity data provide information of the selectivity of nitration sites and structure. The data available at present suggest that tyrosine nitrations in vivo require a specific sequence and structural environment of nitrated proteins.

As pointed out in recent studies [33, 46], the characterization of affinities and binding specificities of monoclonal 3-NT-specific antibodies are of crucial importance and should be ascertained using synthetic tyrosine-nitrated peptides; high antibody affinities were found for nitrotyrosine peptides containing basic (Lys, Arg) amino acid residues in the vicinity of the nitrated tyrosine (Figure 5). Corresponding tyrosine-nitrated peptides may also find important application as biomarkers of oxidative modifications. The observation that 3-NT- specific antibodies will also detect oxidative modifications at tyrosine residues other than nitration, particularly hydroxylations, presents a significant caveat to the use of such antibodies without molecular characterization of affinity-bound proteins and peptides by mass spectrometry. Thus, the combination of both affinity approaches and mass spectrometry will be essential for unequivocal detection, molecular characterization, and quantification of tyrosine nitrations.

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