

Determination of nitration degrees for the birch pollen allergen Bet v 1

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Abstract Nitration of tyrosine residues in the major birch pollen allergen Bet v 1 may alter the allergenic potential of the protein. The kinetics and mechanism of the nitration reaction, however, have not yet been well characterized. To facilitate further investigations, an efficient method to quantify the nitration degree (ND) of small samples of Bet v 1 is required. Here, we present a suitable method of high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD) that can be photometrically calibrated using the amino acids tyrosine (Tyr) and nitrotyrosine (NTyr) without the need for nitrated protein standards. The new method is efficient and in agreement with alternative methods based on hydrolysis and amino acid analysis of tetranitromethane (TNM)-nitrated Bet v 1 standards as well as samples from nitration experiments with peroxynitrite. The results confirm the applicability of the new method for the investigation of the reaction kinetics and mechanism of protein nitration.

Keywords Protein tyrosine nitration · Bet v 1.0101 · HPLC-DAD · Nitration degree

Introduction

Protein nitration can occur during inflammation, oxidative stress, and aging. It has been reported in association with many diseases [1]. Besides endogenous nitration, e.g., by peroxynitrite (ONOO⁻) [1], protein nitration can also occur exogenously in the ambient atmosphere at elevated levels of NO₂ and O₃ (summer smog conditions) [2, 3]. The allergenic potential of plant pollen allergens such as the major birch pollen allergen Bet v 1.0101, termed Bet v 1 hereafter, was found to be enhanced upon nitration [4, 5]. Protein nitration, a stable posttranslational modification (PTM), mainly occurs at tyrosine (Tyr) residues in the polypeptide chain and results in the formation of 3-nitrotyrosine (NTyr).

Several analytical techniques are available to determine the extent of Tyr nitration in proteins: immunochemical techniques [6], high-performance liquid chromatography (HPLC) coupled to ultraviolet–visible (UV-Vis) detection [7, 8], fluorescence detection (FLD) [9], mass spectrometry (MS) [10], or tandem mass spectrometry (MS/MS) [11], and capillary zone electrophoresis (CZE) coupled to MS [12]. However, most of these methods require time-consuming protein hydrolyzation and digestion methods or the use of expensive nitrated protein standards.

Here, we present a simple and efficient method of high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD) for the determination of the nitration degree (ND) of small amounts of the birch pollen allergen Bet v 1. For validation, we compare the new method (HPLC-DAD calibrated with Tyr/NTyr) against three established methods: (1) HPLC-DAD calibrated with nitrated protein standards, (2) protein hydrolysis and amino acid analysis (AAA), and (3)

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protein digestion and liquid chromatography coupled to a tandem mass spectrometer (HPLC-MS/MS).

Materials and methods

Materials

L-3-Nitrotyrosine (NTyr, ALX-106-020-G001), L-tyrosine (Tyr; 93829, 25 g), tetranitromethane (TNM, T25003-5 G), and bicinchoninic acid assay (BCA) kit (BCA1-1 KT) were obtained from Sigma-Aldrich, Schnellendorf, Germany. Acetonitrile (ACN; ROTISOLV HPLC Gradient Grade) and sodium phosphate buffer (10 mM Na₂HPO₄) were obtained from Carl Roth GmbH + Co. KG, Karlsruhe, Germany. Water for chromatography (LiChrosolv), water with 0.1 % (v/v) trifluoroacetic acid (LiChrosolv), and concentrated orthophosphoric acid (H₃PO₄) were obtained from Merck KGaA, Darmstadt, Germany. For all other purposes, high-purity water (18.2 M Ω cm) was taken from an ELGA LabWater system (PURELAB Ultra, ELGA LabWater Global Operations, UK). Recombinant Bet v 1 (Bet v 1.0101) was expressed and purified at the University of Salzburg [13].

Preparation of TNM-nitrated Bet v 1 standards

The protein was dissolved at 1 mg/mL in 10 mM Na₂HPO₄ buffer, adjusted to a pH of 7.45 using concentrated H₃PO₄. TNM was dissolved at a concentration of 0.5 M in MeOH and added to the protein solutions to yield TNM/tyrosine molar ratios of 30/1, 15/1, 5/1, and 1/1. Reaction mixtures were stirred for 60 min at room temperature (RT). For each experiment, a mock-control protein sample was treated in the same way, but without the addition of TNM (MeOH only). After the reaction was complete, samples were centrifuged through a centrifugal filter device (Amicon centrifuge tube; Merck Millipore, Cork, Ireland) and rinsed twice with fresh 10 mM Na₂HPO₄ buffer to remove TNM and MeOH. The samples were analyzed by amino acid analysis and served as calibration standards.

Nitration of Bet v 1 by peroxyntirite (ONOO⁻)

Samples containing Bet v 1 were re-buffered in 50 mM NH₄HCO₃ buffer (pH=7.8), with or without the addition of 0.1 mM diethylenetriamine pentaacetic acid (DTPA) (Sigma-Aldrich, St. Louis, MO, USA). Sodium peroxyntirite (Cedarlane, Ontario, Canada) was added, after being thawed on ice, to yield ONOO⁻/Tyr molar ratios of 30/1, 15/1, 5/1, and 1/1. Different reaction conditions were tested: reaction times of 15 and 100 min, temperatures of 4 °C and RT, and in the presence or absence of DTPA. Reaction mixtures were stirred and samples were centrifuged through a centrifugal

filter device (Amicon centrifuge tube (10 kDa); Merck Millipore, Cork, Ireland) and rinsed twice with fresh 10 mM Na₂HPO₄ buffer.

HPLC-DAD analysis and calibration

The HPLC-DAD system (Agilent Technologies 1200 series) consisted of a binary pump (G1379B), an autosampler with thermostat (G1330B), a column thermostat (G1316B), and a photodiode array detector (DAD, G1315C). ChemStation software (Rev. B.03.01, Agilent) was used for system control and data analysis. A monomerically bound C₁₈ column (Vydac 238TP, 250 mm×2.1 mm inner diameter, 5 μ m particle size; Grace Vydac, Alltech) was used for chromatographic separation. Eluents were 0.1 % (v/v) trifluoroacetic acid in water (eluent A) and ACN (eluent B). Gradient elution was performed at a flow rate of 200 μ L/min. For each chromatographic run, the solvent gradient started at 3 % B followed by a linear gradient to 90 % B within 15 min, flushing back to 3 % B within 0.2 min, and maintaining 3 % B for additional 2.8 min. Column re-equilibration time was 5 min before the next run. Absorbance was monitored at wavelengths of 280 and 357 nm. The sample injection volume was 10–80 μ L. Each chromatographic run was repeated three times.

The ND is determined according to Eq. 4. The derivation of the equation is as follows:

The ND is defined as the concentration (c in moles per liter) of NTyr divided by the sum of the concentrations of NTyr and Tyr (Eq. 1):

$$ND = \frac{c_{NTyr}}{c_{NTyr} + c_{Tyr}} \quad (1)$$

Tyr and NTyr quantification is based on spectrophotometry. Both Tyr and NTyr show absorption maxima at 280 nm, while NTyr exhibits an additional absorption maximum at 357 nm in acidic solution (0.05 M acetic acid, pH~3.5) [7, 8]. According to the Beer–Lambert law, the concentration of a species (x) is proportional to the absorbance (A in absorbance units, AU) at a wavelength (λ in nanometer) in the range of linearity ($0.05 < A < 1$):

$$c_x = \frac{A_{x,\lambda}}{\varepsilon_{x,\lambda} \times l} \quad (2)$$

where $\varepsilon_{x,\lambda}$ is the molar extinction coefficient (in liters per mole-centimeter) and l (in centimeter) is the optical path length. As Tyr and NTyr are measured simultaneously by the HPLC-DAD method (intact proteins), knowledge of the relative contribution of each amino acid to the absorbance signal is necessary for the calculation of the ND according to

Eq. 2. Thus, scaling factors (k and f) are needed to scale the extinction coefficients (ε):

$$f = \frac{\varepsilon_{NTyr,280}}{\varepsilon_{Tyr,280}}, k = \frac{\varepsilon_{NTyr,280}}{\varepsilon_{NTyr,357}}, \frac{f}{k} = \frac{\varepsilon_{NTyr,357}}{\varepsilon_{Tyr,280}} \quad (3)$$

Reformulation of Eqs. 1 and 2, and consideration of the scaling factors k and f (Eq. 3), leads to Eq. 4, which describes the ND of a mixture of Tyr and NTyr:

$$ND = \frac{A_{NTyr,357}}{A_{NTyr,357} + \frac{f}{k} (A_{all,280} - k A_{NTyr,357})} \quad (4)$$

Determination of scaling factors k and f by UV spectrophotometry

UV absorbance measurements of amino acid standard solutions were carried out on a Lambda 25 UV/Vis spectrophotometer (PerkinElmer, Waltham, MA, USA). Different concentrations (0.069, 0.138, and 0.276 mM) of Tyr and NTyr in 50 mM acetic acid (pH~3) were measured (18 replicates each) at A_{280} and A_{357} , respectively, and the scaling factors k and f were determined graphically (see Electronic Supplementary Material Fig. S1). Scaling factor $k=1.79\pm0.06$ (linear regression, $n=3$) was determined by plotting the absorbance signal of NTyr at 280 nm ($A_{NTyr,280}$) against the absorbance signal of NTyr at 357 nm ($A_{NTyr,357}$) (see Electronic Supplementary Material Fig. S1a). Scaling factor $f=2.93\pm0.06$ (linear regression, $n=3$) was determined by plotting the absorbance signal of NTyr at 280 nm ($A_{NTyr,280}$) against the absorbance signal of Tyr at 280 nm ($A_{Tyr,280}$) (see Electronic Supplementary Material Fig. S1b). It should be noted that NTyr can form an internal hydrogen bond between the nitro and the phenolic hydroxyl groups, causing its absorption properties to be pH-dependent, with A_{280} increasing and A_{357} decreasing with decreasing pH [15]. We also found the scaling factors k and f to be pH-dependent. Nevertheless, the scaling factors k and f , determined by UV photometry as described above (pH~3), reflect the absorption properties of NTyr in intact Bet v 1 proteins during the HPLC-DAD analysis (pH~2), as shown below by the agreement with alternative methods.

Alternative analytical methods

Experimental details about the alternative methods applied to validate the newly developed HPLC-DAD method are specified in the [Electronic Supplementary Material](#), sections 1–3.

Results

The HPLC-DAD method developed in this study enables efficient determination of protein NDs based on photometric calibration using the amino acids tyrosine (Tyr) and nitrotyrosine (NTyr) without the need for nitrated protein standards. To validate the new method, we compared the ND results with three alternative methods for the determination of the total protein ND: (1) HPLC-DAD analysis calibrated using TNM-nitrated Bet v 1 standards, (2) AAA, and (3) HPLC-MS/MS analysis. For the TNM-nitrated Bet v 1 standards, all three methods agree well (Fig. 1) with slopes close to 1 and high correlation coefficients, which confirm the applicability and consistency of the new HPLC-DAD method for Bet v 1. With regard to the ND, the limit of detection was found to be 0.01, determined by nine consecutive HPLC-DAD measurements of unmodified Bet v 1 using the 3σ method. Additionally, the HPLC-DAD analysis is more robust, in terms of the standard deviations of the analytical replicates, compared with AAA and HPLC-MS/MS measurements, which we attribute to the simpler sample preparation (no hydrolysis or digestion of the protein).

Nitration reactions with $ONOO^-$ as the nitrating reagent are more complex than those carried out using TNM and will also result in increased oxidation and oxidative degradation of the protein (lower recovery). Figure 2 shows the results of a method comparison similar to the previous section. Again, the new spectrophotometrically calibrated HPLC-DAD method was compared to (1) the HPLC-DAD method calibrated with TNM-nitrated Bet v 1 standards (Fig. 2a), (2) AAA (Fig. 2b), and (3) HPLC-MS/MS analysis (Fig. 2c). While both calibration methods for the HPLC-DAD analysis and AAA agree well with slopes close to 1 and high correlation coefficients ($R^2=0.999$ and $R^2=0.837$, respectively), the HPLC-MS/MS analyses seem to underestimate the ND of the protein (slope of 0.637 ± 0.192). However, due to the large scatter in the HPLC-MS/MS data, also the correlation coefficient with the new HPLC-DAD method, $R^2=0.264$, is low. The large scatter in the HPLC-MS/MS is likely due to oxidative degradation of the protein during the reaction with $ONOO^-$ [14], which leads to the formation of peptide fragments other than those expected for the tryptic digestion of an intact protein and may also enhance ion suppression during mass spectrometric detection. Therefore, the spectrophotometric detection appears more robust for the analysis of $ONOO^-$ nitrated proteins compared to the mass spectrometric detection.

It should be noted that Bet v 1 does not contain tryptophan residues, which might influence measurement results due to an absorption band of tryptophan at 280 nm ($\varepsilon=5,540$ L/(mol cm), pH=7.0, [16]). This potential interference has to be considered when applying the new calibration method for determining the ND of tryptophan-containing proteins. Oxidation of tryptophan may also contribute to light absorption at

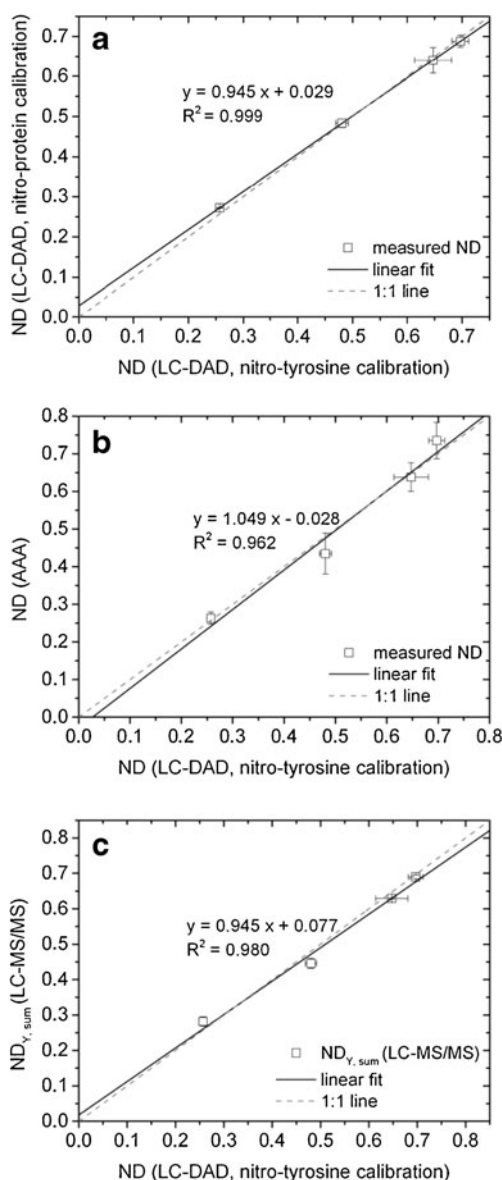


Fig. 1 Method comparison for TNM-nitrated Bet v 1 standards. **a** NDs from HPLC-DAD using the calibration with nitro-protein standards are plotted against the NDs from HPLC-DAD calibrated with NTyr. **b** NDs from AAA are plotted against the NDs from HPLC-DAD calibrated with NTyr. **c** $ND_{Y, sum}$ values from HPLC-MS/MS measurements summing up the individual NDs are plotted against the NDs from HPLC-DAD calibrated with NTyr. The error bars indicate the standard deviation of three analytical replicates. The 1:1 line is dashed

~360 nm, but absorbances measured at this wavelength have already been successfully applied for the detection of tyrosine nitration in earlier studies ([17] and references therein). Experiments with ovalbumin nitrated by peroxynitrite show that the new method presented here is also applicable to this protein which contains three tryptophan residues (see Electronic Supplementary Material Fig. S2).

Overall, our results show that a simple HPLC-DAD method can be used for efficient determination of the nitration

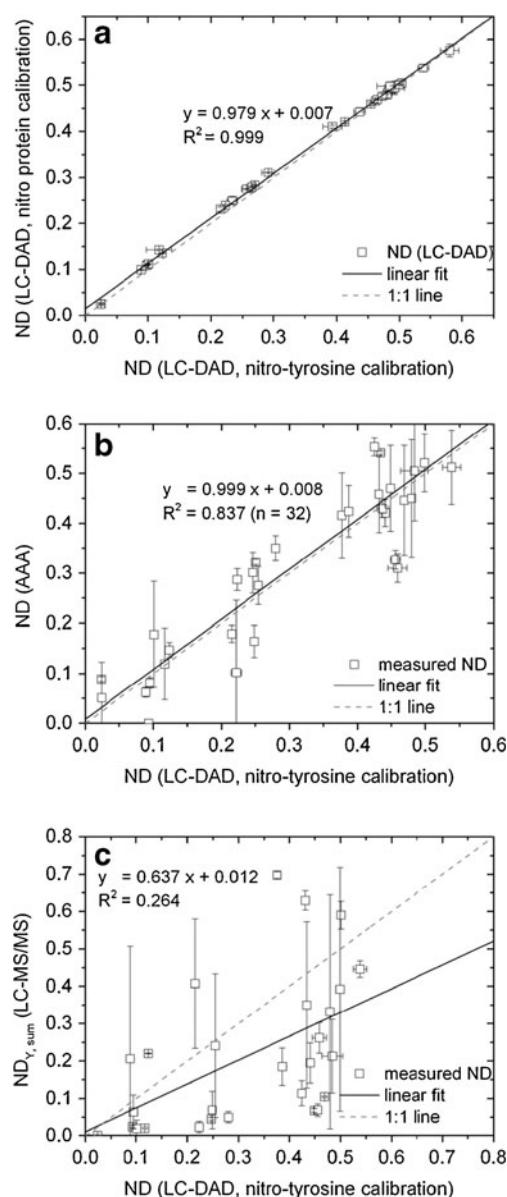


Fig. 2 Method comparison for Bet v 1 nitrated by peroxynitrite. **a** NDs from HPLC-DAD using the calibration with nitro-protein standards are plotted against the NDs from HPLC-DAD calibrated with NTyr. **b** NDs from AAA are plotted against the NDs from HPLC-DAD calibrated with NTyr. **c** $ND_{Y, sum}$ values from HPLC-MS/MS measurements summing up the individual NDs are plotted against the NDs from HPLC-DAD calibrated with NTyr. The error bars indicate the standard deviation of three analytical replicates. The 1:1 line is dashed

degree of Bet v 1 and other intact proteins. No enzymatic digestion or hydrolyzation of the investigated protein is necessary, and the laborious and expensive preparation of nitrated protein standards can be avoided by photometric calibration with the amino acids of interest, i.e., Tyr and NTyr. The new method provides a simple and efficient way of investigating the reaction kinetics and mechanism of protein nitration by different nitrating agents and conditions to be pursued in follow-up studies.

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References

- Greenacre SAB, Ischiropoulos H (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* 34(6):541–581. doi:10.1080/10715760100300471
- Shiraiwa M, Sosedova Y, Rouviere A, Yang H, Zhang YY, Abbatt JPD, Ammann M, Pöschl U (2011) The role of long-lived reactive oxygen intermediates in the reaction of ozone with aerosol particles. *Nat Chem* 3(4):291–295. doi:10.1038/nchem.988
- Franze T, Weller MG, Niessner R, Pöschl U (2005) Protein nitration by polluted air. *Environ Sci Technol* 39(6):1673–1678. doi:10.1021/es0488737
- Gruijthuijsen YK, Grieshuber I, Stöcklinger A, Tischler U, Fehrenbach T, Weller MG, Vogel L, Vieths S, Pöschl U, Duschl A (2006) Nitration enhances the allergenic potential of proteins. *Int Arch Allergy Immunol* 141(3):265–275. doi:10.1159/000095296
- Karle AC, Oostingh GJ, Mutschlechner S, Ferreira F, Lackner P, Bohle B, Fischer GF, Vogt AB, Duschl A (2012) Nitration of the pollen allergen Bet v 1.0101 enhances the presentation of Bet v 1-derived peptides by HLA-DR on human dendritic cells. *PLoS One* 7(2):e31483
- Franze T, Weller MG, Niessner R, Pöschl U (2003) Enzyme immunoassays for the investigation of protein nitration by air pollutants. *Analyst* 128(7):824–831. doi:10.1039/b303132b
- Crow JP, Beckman JS (1995) Quantitation of protein tyrosine, 3-nitrotyrosine, and 3-aminotyrosine utilizing HPLC and intrinsic ultraviolet absorbance. *Methods (Orlando)* 7(1):116–120. doi:10.1006/meth.1995.1017
- Yang H, Zhang YY, Pöschl U (2010) Quantification of nitrotyrosine in nitrated proteins. *Anal Bioanal Chem* 397(2):879–886. doi:10.1007/s00216-010-3557-3
- Kamisaki Y, Wada K, Nakamoto K, Kishimoto Y, Kitano M, Itoh T (1996) Sensitive determination of nitrotyrosine in human plasma by isocratic high-performance liquid chromatography. *J Chromatogr B-Biomed Appl* 685(2):343–347. doi:10.1016/s0378-4347(96)00202-2
- Walcher W, Franze T, Weller MG, Pöschl U, Huber CG (2003) Liquid- and gas-phase nitration of bovine serum albumin studied by LC-MS and LC-MS/MS using monolithic columns. *J Proteome Res* 2(5):534–542. doi:10.1021/pr034034s
- Zhang YY, Yang H, Pöschl U (2011) Analysis of nitrated proteins and tryptic peptides by HPLC-chip-MS/MS: site-specific quantification, nitration degree, and reactivity of tyrosine residues. *Anal Bioanal Chem* 399(1):459–471. doi:10.1007/s00216-010-4280-9
- Gusenkov S, Ackaert C, Stutz H (2013) Separation and characterization of nitrated variants of the major birch pollen allergen by CZE-ESI- μ TOF MS. *Electrophoresis*. doi:10.1002/elps.201300151
- Kofler S, Asam C, Eckhard U, Wallner M, Ferreira F, Brandstetter H (2012) Crystallographically Mapped Ligand Binding Differs in High and Low IgE Binding Isoforms of Birch Pollen Allergen Bet v 1. *J Mol Biol* 422(1):109–123
- Ferrer-Sueta G, Radi R (2009) Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. *ACS Chem Biol* 4(3):161–177. doi:10.1021/cb800279q
- De Filippis V, Frasson R, Fontana A (2006) 3-Nitrotyrosine as a spectroscopic probe for investigating protein-protein interactions. *Protein Sci* 15(5):976–986. doi:10.1110/ps.051957006
- Mach H, Middaugh CR, Lewis RV (1992) Statistical determination of the average values of the extinction coefficients of tryptophan and tyrosine in native proteins. *Anal Biochem* 200(1):74–80. doi:10.1016/0003-2697(92)90279-g
- Abello N, Kerstjens HAM, Postma DS, Bischoff R (2009) Protein tyrosine nitration: selectivity, physicochemical and biological consequences, denitration, and proteomics methods for the identification of tyrosine-nitrated proteins. *J Proteome Res* 8(7):3222–3238. doi:10.1021/pr900039c