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Nitration of the Birch Pollen Allergen Bet v 1.0101: Efficiency and Site-Selectivity of Liquid and Gaseous Nitrating Agents

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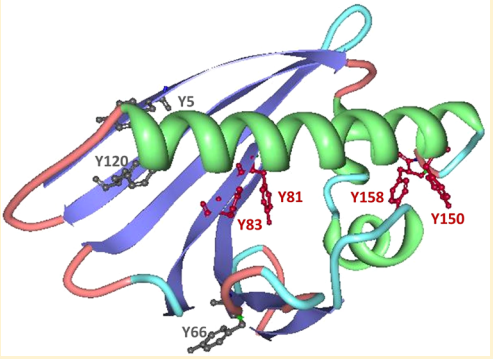
Supporting Information

ABSTRACT: Nitration of the major birch pollen allergen Bet v 1 alters the immune responses toward this protein, but the underlying chemical mechanisms are not yet understood. Here we address the efficiency and site-selectivity of the nitration reaction of recombinant protein samples of Bet v 1.0101 with different nitrating agents relevant for laboratory investigations (tetranitromethane, TNM), for physiological processes (peroxynitrite, $ONOO^-$), and for the health effects of environmental pollutants (nitrogen dioxide and ozone, O_3/NO_2). We determined the total tyrosine nitration degrees (ND) and the NDs of individual tyrosine residues (ND_Y). High-performance liquid chromatography coupled to diode array detection and HPLC coupled to high-resolution mass spectrometry analysis of intact proteins, HPLC coupled to tandem mass spectrometry analysis of tryptic peptides, and amino acid analysis of hydrolyzed samples were performed. The preferred reaction sites were tyrosine residues at the following positions in the polypeptide chain: Y83 and Y81 for TNM, Y150 for $ONOO^-$, and Y83 and Y158 for O_3/NO_2. The tyrosine residues Y83 and Y81 are located in a hydrophobic cavity, while Y150 and Y158 are located in solvent-accessible and flexible structures of the C-terminal region. The heterogeneous reaction with O_3/NO_2 was found to be strongly dependent on the phase state of the protein. Nitration rates were about one order of magnitude higher for aqueous protein solutions (~20% per day) than for protein filter samples (~2% per day). Overall, our findings show that the kinetics and site-selectivity of nitration strongly depend on the nitrating agent and reaction conditions, which may also affect the biological function and adverse health effects of the nitrated protein.



KEYWORDS: *Bet v 1.0101, HPLC−MS/MS, tyrosine nitration, nitration sites, air pollution*

1. INTRODUCTION

Post-translational modifications such as nitration and oxidation of proteins can occur during inflammation, oxidative stress, and chemical aging under physiological or environmental conditions. Protein nitration has been reported in association with at least 50 different diseases,[1,2] and it has been shown to alter the immunogenic potential of food allergens[3] and aeroallergens like Bet v 1.[4,5]

Numerous studies have suggested that asthma and allergic diseases are enhanced by traffic-related air pollution with high concentrations of nitrogen oxides (NO_x) and ozone (O_3).[6,7] Nitration reactions and related changes in the immunogenicity of allergenic proteins might explain the promotion of allergies by traffic-related air pollution.[8] Recently, it has been shown that birch pollen from urban areas had a higher allergenic potential than pollen from rural areas, although the allergen content remained unchanged,[9] and ragweed pollen collected along roads with heavy traffic showed a higher allergenic potential compared with pollen collected in remote areas.[10]

The standard nitrating agent in laboratory studies of protein chemistry is tetranitromethane (TNM), which selectively nitrates tyrosine residues. The reaction conditions are mild: nitration is performed at neutral pH, low ionic strength, and room temperature.[11] The nitration mechanism may either involve ionic species, formed from a partial dissociation of TNM into nitronium ions and nitroformate ions, or radical species.[11−13]

Under physiological conditions, the reaction of free nitrogen oxide radicals (NO) and superoxide anions (O_2^-) can form

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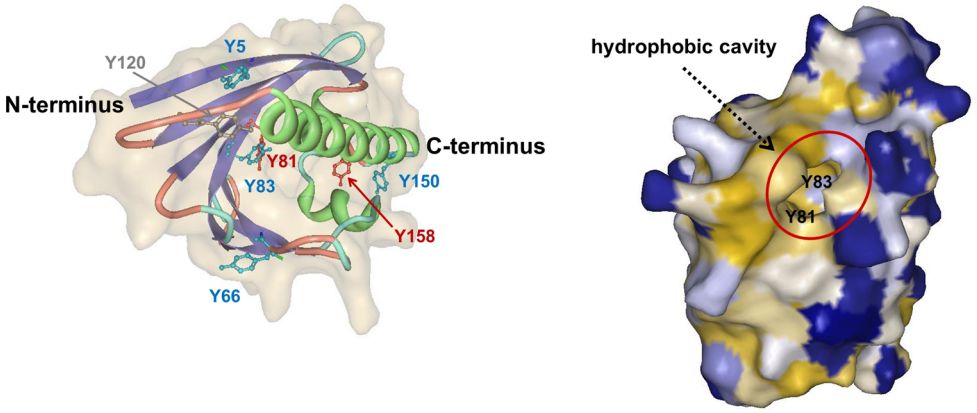

Figure 1. Position of tyrosine residues in the 3-D structure of crystallized unmodified Bet v 1.0101 (PDB accession code: 4A88)[28] created with the PDB protein workshop 3.9.[44] (left) Tyr residues indicated in blue (Y5, Y66, Y83, and Y150) have approximate values of solvent exposure of >20%, the red-indicated residues (Y81 and Y158) have values above 15%, and the gray-indicated Tyr residue Y120 has a value below 5%.[32] Y150 and Y158 are located in solvent-accessible and flexible structures of the C-terminal region of the protein. (right) Surface area (indicating very hydrophobic surfaces in dark yellow, slightly hydrophobic surfaces in light yellow, polar surfaces in light blue, and charged surfaces in dark blue)[45] of Bet v 1.0101 with the hydrophobic cavity. Y81 and Y83 are inside the hydrophobic cavity.

strong oxidizing and nitrating intermediates, such as peroxynitrite ($ONOO^-$), which is also able to nitrate tyrosine residues in proteins, leading to the formation of 3-nitrotyrosine.[14−16]

In a first step, tyrosine is oxidized involving free radical mechanisms in which one-electron oxidants derived from $ONOO^-$ (OH, NO_2, and carbonate radicals CO_3^-) attack the aromatic ring, leading to the formation of a Tyr radical. In a second step, the Tyr radical combines with NO_2 to yield 3-nitrotyrosine. Possible side reactions include the formation of protein dimers by cross-linking of Tyr radicals.[17−20]

In addition, proteins can be modified in the atmosphere when exposed to pollutants such as nitrogen dioxide (NO_2) and ozone (O_3). Laboratory and field studies showed that proteins were efficiently nitrated upon exposure to gas mixtures of NO_2 and O_3 or polluted urban air (summer smog).[21] The heterogeneous reaction of the protein with the gaseous reactants O_3 and NO_2 was found to form nitrated and oxidized proteins as well as protein degradation and aggregation products.[22] Recent studies found a higher nitration degree (ND) when the protein was pretreated with O_3. The conclusion of these studies was that the nitration reaction of proteins with O_3 and NO_2 proceeds through long-lived reactive oxygen intermediates (ROIs).[23] The protein first reacts with O_3 and forms a ROI, most likely Tyr radicals, which can persist over extended periods of time. In a second step, the ROI reacts with NO_2, resulting in the formation of 3-nitrotyrosine residues. This two-step mechanism, including an oxidation step and an NO_2 addition step, is comparable to the nitration mechanism of $ONOO^-$. In the case of $ONOO^-$ nitration, the oxidants for the first step are CO_3^- radicals and oxo-metal complexes or, to a lesser extent, OH and NO_2 radicals, while for the heterogeneous reaction with the gaseous components O_3 is the oxidizing agent. The second step in both cases is the addition of either exogenous or endogenous NO_2. Again, the dimerization of Tyr radicals competes with the formation of 3-nitrotyrosine.

The recombinant protein Bet v 1.0101 has a molecular mass of 17.44 kDa and consists of 159 amino acids including seven tyrosine (Tyr) residues: Y5, Y66, Y81, Y83, Y120, Y150, and Y158. The 3-D structure of Bet v 1.0101 is shown in Figure 1.

In the presented study, we address the efficiency and site-selectivity for the reaction of recombinant Bet v 1.0101, simply termed Bet v 1 hereafter, with the standard laboratory reagent for protein nitration (TNM) and two naturally occurring nitrating reagents, that is, $ONOO^-$ mimicking inflammation and oxidative/nitrosative stress and O_3 in combination with NO_2 mimicking air pollution effects. High-performance liquid chromatography coupled to diode array detection (HPLC-DAD) analysis of intact protein samples and amino acid analysis (AAA) of hydrolyzed protein samples were utilized to determine the total tyrosine ND in nitrated Bet v 1 samples.[24,25] Additionally, HPLC coupled to tandem mass spectrometry (MS/MS) analysis of tryptic peptides was used to elucidate reaction mechanisms and site-specific nitration patterns.[26,27] Further insights into protein modification were obtained by HPLC coupled to high-resolution mass spectrometry (HR-MS) analysis.

2. MATERIALS AND METHODS

Protein Production and Purification

Bet v 1 was expressed in *E. coli* during an incubation time of 4 h at 37 °C and purified as previously described[28] with minor modifications. The natural origin of the protein is *Betula verrucosa*, and the origin of the recombinant is *E. coli* (strain BL21 (DE)3). The clone's accession numbers are Genbank X15877 and Uniprot P15494. The cell pellets were dissolved in 25 mM imidazole buffer containing 0.1% Triton-X100, pH 7.4, including one tablet of complete EDTA-free protease inhibitor (no. 04 693 132 001, Roche). Thereafter, the cells were frozen and thawed three times, and DNase digestion was followed by acidic salt precipitation overnight. Three subsequent purification methods were used: hydrophobic interaction chromatography (HIC), ion exchange chromatography (IEX), and size-exclusion chromatography (SEC). After every purification step, the fractions were loaded on an SDS-gel, and only the purest fractions were pooled and used for the next steps. The yield of the expression and purification of Bet v 1.0101 is ~50 mg Bet v 1 per liter culture. The concentration of working solution (after purification) is 1 mg/mL. The buffer is a salt-free 10 mM PO_4 buffer with pH 7.4.

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Nitration with TNM

Bet v 1 was nitrated as previously described.[24] In brief, TNM (T25003-5 G, Sigma-Aldrich) was dissolved at a concentration of 0.5 M in MeOH and added to the protein solutions (1 mg mL^{-1}, 10 mM Na_2HPO_4, Carl Roth, Karlsruhe, Germany) to yield TNM/tyrosine molar ratios of 30/1, 15/1, 5/1, and 1/1. By adding additional amounts of MeOH, the final MeOH concentrations were kept constant for each molar ratio. Reaction mixtures were stirred for 60 min at room temperature (RT). The reaction was stopped by centrifugation through an Amicon centrifugal device with a 10 kDa cutoff membrane (Merck Millipore, Cork, Ireland).

Nitration with $ONOO^-$

Samples containing Bet v 1 were rebuffered in a 50 mM NH_4HCO_3 buffer (pH 7.8), with or without the addition of 0.1 mM diethylenetriamine pentaacetic acid (DTPA) (Sigma-Aldrich, St. Louis, MO) to a final allergen concentration of 1.0 mg mL^{-1}, as previously described.[24] Sodium peroxynitrite (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 the presence or absence of DTPA. The reaction was stopped by centrifugation through an Amicon centrifugal device with a 10 kDa cutoff membrane. The half life of $ONOO^-$ under physiological conditions is <1 s, complicating the determination of the actual $ONOO^-$ concentrations to which proteins are exposed.[14] Peroxynitrite is stable under alkaline aqueous solutions, but it decomposes at a lower pH, where the peroxynitrite anion is protonated and forms peroxynitrous acid (ONOOH), which can form hydroxyl radicals (OH) and nitrogen dioxide radicals (NO_2).[29] Accordingly, the reaction is more difficult to control than the nitration reaction using TNM. The experimental conditions are listed in the Supporting Information (Table S1).

Nitration with O_3/NO_2

Bet v 1 was exposed to O_3/NO_2 mixtures. For details on the experimental setup and conditions used to investigate the nitration efficiency of Bet v 1 at varying relative humidity (RH) and protein phase state, we refer to Supporting Information Section S3, Table S2, and Figure S1. In brief, the heterogeneous reaction between Bet v 1 and gaseous reactants was studied using protein-loaded (precleaned) syringe filters, which were exposed to O_3/NO_2 mixtures at different RH. Additionally, the homogeneous reaction of the dissolved protein and the reactants was studied by bubbling O_3/NO_2 directly through an aqueous solution of Bet v 1.

Bradford Assay and Bicinchoninic Acid Assay

The approximate protein concentration was determined against a standard curve made with native Bet v 1 using the Bradford assay and with the bicinchoninic acid (BCA, BCA1-1 KT, Sigma-Aldrich) assay using a bovine serum albumin (BSA) protein standard obtained from Sigma Aldrich.

Amino Acid Analysis

The samples were analyzed by AAA using the Pico-Tag method (Waters, Milford, MA) according to the manufacturer's instructions, as detailed in Selzle and Ackaert et al.[24] In brief, the protein samples were analyzed by reversed-phase HPLC on a HP110 system (Hewlett-Packard, San Jose, CA) after total hydrolysis and phenylisothiocyanate derivatization. Data were collected using the Chemstation software. Total NDs were calculated based on the quantification of tyrosine and 3-nitrotyrosine derivatives.

HPLC–DAD Analysis

The protein solutions were analyzed using an HPLC–DAD system (Agilent Technologies 1200 series), as previously described.[24]

HPLC–MS/MS Analysis

Tryptic peptides were analyzed using an HPLC–Q-TOF instrument (Agilent Technologies 1200 series coupled to Agilent Technologies 6520 Accurate-Mass Q-TOF) as previously described[24] and detailed in the Supporting Information Section S1.

The individual nitration degree (ND_Y) of a specific Tyr residue (Y) is defined as the intensity of the nitrated peptide divided by the sum of the intensities of the nitrated and unmodified peptides as described elsewhere.[27] The calculation of ND_Y was based on the observation that the ionization efficiencies for unmodified and nitrated synthesized peptides were found to be similar with a mean difference of 0.17 ± 0.12 (arithmetic mean ± standard deviation, n = 3; see Table S3 in the Supporting Information). Thus, (semi)quantitative information about the site selectivity of the nitration reaction could be obtained. The overall ND was calculated from the ND_Y data by summing the ND_Ys and dividing the sum by the Tyr coverage and the number of Tyr residues per molecule and showed a good correlation with alternative methods.[24]

For the identification of the nitration sites, exemplary MS/MS spectra are shown in Figures S2–S9 in the Supporting Information for each nitrating agent. The Figures include peptide sequence, precursor mass, charge state, MH^+ error, and search engine scores. In some cases, the distinction of the nitration site in the mononitrated peptide YNYVIEGGPGDTLEK (AA 81–95) was not possible due to equal scores for Y81 and Y83. The signals at m/z 278.1 (b_2 for unmodified Y81 and $b_4^{+2}{}_{-H_2O}$ for modified Y81) and m/z 321.1 (b_2 for nitrated Y81 and $b_7^{+2}{}_{-H_2O}$ for nitrated Y83) could not be attributed unambiguously. The MS/MS spectra were reviewed manually for the presence of the immonium ions of tyrosine (m/z 136.1) and nitro-tyrosine (m/z 181.1). Distinction of the mononitration of Y81 or Y83 was possible in the case of sufficient spectra information and different scores.

HPLC–HR-MS Analysis

The protein solutions were separated with a capillary HPLC system (Model UltiMate3000, Dionex Benelux), and high-resolution (HR) mass analysis was performed with an Orbitrap mass spectrometer (Model Exactive, ThermoFisher Scientific) under optimized conditions, as previously published.[30] The mass spectra were analyzed using the data evaluation software Xcalibur (Thermo Scientific) and the implemented deconvolution tool Xtract. For isotopically unresolved mass spectra, the software ProMass (ThermoFisher Scientific) was utilized to calculate the average molecular mass of the intact protein. Analysis of the intact protein by HPLC–HR-MS was used to elucidate modifications of the protein by detection of molecular masses. It allowed relative quantification of the unmodified protein, different nitration states of the protein, and other modifications, for example, oxidation, degradation, and aggregation. For details, see the Supporting Information Section S2.

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Table 1. Most Frequent Tryptic Peptides Identified by LC−MS/MS Analysis Are Shown Exemplarily as Peptides from the Reaction of Bet v 1 with 5 Molar Excess of $ONOO^-$[a]

AA	Tyr position	sequence	RT (min)	m/z measured	MH^+ (matched)
21−32		AFILDGDNLFPK	20.24	675.3562	1349.71
33−54		VAPQAISSVENIEGNGGPGTIK	16.44	713.3744	2138.1088
33−55		VAPQAISSVENIEGNGGPGTIKK	15.81	756.0684	2266.2038
55−65		KISFPEGFPFK	19.04	648.8489	1296.6987
56−65		ISFPEGFPFK	20.34	584.8066	1168.6037
56−68	**66**	ISFPEGFPFK**Y**VK	21.90	535.2755	1558.83
69−80		DRVDEVDHTNFK	11.76	369.4242	1474.6921
71−80		VDEVDHTNFK	10.69	401.86	1203.564
81−97	81, 83	YNYSVIEGGPIGDTLEK	17.56	927.9635	1854.912
98−103		ISNEIK	2.80	352.1994	703.3985
98−115		ISNEIKIVATPDGGSILK	17.63	619.0197	1855.054
104−115		IVATPDGGSILK	14.92	585.8395	1170.6729
146−159	150, 158	AVESYLLAHSDAYN	16.31	776.8688	1552.7278
146−159	**150**	AVES**Y**LLAHSDAYN	17.69	799.3589	1552.7278
146−159	**158**	AVESYLLAHSDA**Y**N	17.76	533.2367	1552.7278
146−159	**150, 158**	AVES**Y**LLAHSDA**Y**N	19.13	821.8481	1552.7278

[a]Amino acid (AA) numbers, Tyr positions (bold = modified by nitration, +45 Da), peptide sequence, retention time (RT), and the measured and matched mass signals are shown.

3. RESULTS AND DISCUSSION

The nitration efficiency and site-selectivity of recombinant Bet v 1 nitrated by different nitrating agents was studied in a total of 118 experiments. Bet v 1 was nitrated in aqueous solution by TNM, $ONOO^-$, and O_3/NO_2, and heterogeneously via exposure of Bet v 1 filter samples to gaseous O_3/NO_2 mixtures. For the water-soluble nitration products, we determined total NDs averaged over all tyrosine residues in the protein and site-specific NDs of individual tyrosine residues (ND_Y) in the protein by different chromatographic techniques: HPLC-DAD and HPLC-HR-MS of intact proteins, HPLC−MS/MS of tryptic peptides, and AAA of hydrolyzed samples. In Table 1 exemplary tryptic peptides derived from the HPLC−MS/MS analysis are shown. The Tyr residues are located at the following peptides: Y5 (GVFNYETTSVIPAAR), Y66 (YVK or ISFPEGFPFKYVK), Y81 + Y83 (YNYSVIEGGPIGDTLEK), Y120 (YHTK), and Y150 + Y158 (AVESYLLAHSDAYN).

Nitration with TNM

Figure 2 summarizes the results of the nitration of Bet v 1 in aqueous solution with different amounts of TNM. Increasing the molar ratio of TNM over tyrosine (TNM/Y) resulted in increasing NDs for the soluble fraction of the intact protein, with NDs ranging from 23.6 to 72.3% (Figure 2a). All three methods used to determine the ND in Bet v 1 (AAA, HPLC−DAD, HPLC−MS/MS) were found to agree well within the measurement uncertainties.

Figure 2b shows ND_Y as a function of TNM/Y, derived from the relative quantification of unmodified and nitrated peptides by HPLC−MS/MS. (For details, see Supporting Information S1.) The amino acid sequence coverage was (64.5 ± 1.7)% (arithmetic mean ± standard deviation), and four of seven tyrosine residues in Bet v 1 were detected. The peptides containing Y5, Y66, and Y120 could not be detected in any of the runs. In particular, Y66 and Y120, which are located at short peptides, were difficult to identify reliably due to insufficient spectra information. Y83 and Y81 were the most preferred nitration sites, and Y83 was found in the nitrated state only, even at low total ND (Table 2). Hydroxylation of tyrosine or phenylalanine or the oxidation of methionine was not observed in the HPLC−MS/MS analysis.

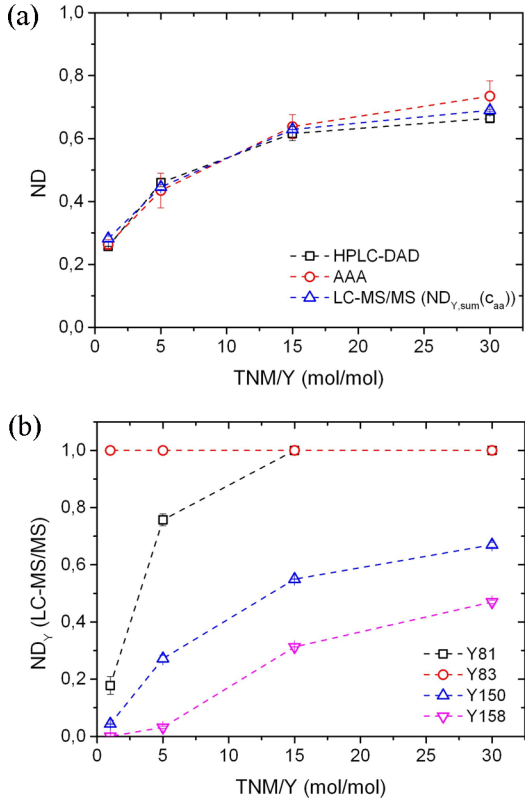

Figure 2. Nitration degrees for dissolved Bet v 1 nitrated by tetranitromethane (TNM) in solution plotted against the molar ratio of nitrating agent and tyrosine residues (TNM/Y): (a) total nitration degrees (ND) determined by AAA, HPLC−DAD, and LC−MS/MS and (b) site-specific nitration degrees (ND_Y) determined by LC−MS/MS. Data points and error bars represent the arithmetic mean values and standard errors of four chromatographic runs.

HPLC−HR-MS results also show an increasing number of nitro groups added to the protein at increasing TNM/Y ratios (mass shift Δm = 45 Da). Additionally, oxidation, which is

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Table 2. Specific Nitration Degrees for Individual Tyrosine Residues Determined by LC−MS/MS for Nitration in Aqueous Solution by TNM (ND = 26%), $ONOO^-$ (ND = 25%), and O_3/NO_2 (ND = 20%) and for the Heterogeneous Reaction with O_3/NO_2 (ND = 4%)[a]

Tyr position	secondary structure	TNM ND_Y (%)[b]	$ONOO^-$ ND_Y (%)[c]	O_3/NO_2 ND_Y (%)[d]	O_3/NO_2 ND_Y (%)[e]
Y5	β-strand				
Y66	β-strand				
Y81	β-strand	18 ± 5	20 ± 9[f]	12 ± 4[f]	2 ± 2
Y83	β-strand	100 ± 0			47 ± 12
Y120	β-strand				
Y150	α-helix	4 ± 0.1	57 ± 16	5 ± 1	0 ± 0
Y158	coil	0 ± 0	6 ± 8	20 ± 1	49 ± 12

[a]The reported data are arithmetic mean values and standard errors for two to four replicates, as specified in the table footnotes. Results of exemplary HPLC−MS/MS measurements are shown and represent only the specified reaction conditions. [b]Reaction in aqueous solution with TNM/Y = 1 with a reaction time of 60 min, n = 4 (analytical replicates). [c]Reaction in aqueous solution with $ONOO^-$/Y = 5 with a reaction time of 15 min (experiment 16 + 17, Table S1 in the Supporting Information), n = 2 (experimental replicates). [d]Reaction in aqueous solution with 100 ppb O3 and 100 ppb NO2 with a reaction time of 17 h (experiment 11, Table S2 in the Supporting Information), n = 4 (experimental replicates). [e]Heterogeneous reaction of protein on filter with 230 ppb O_3 and 230 ppb NO_2 with a reaction time of 48 h (experiment 16, Table S2 in the Supporting Information), n = 4 (experimental replicates) [f]Y81 and Y83 not clearly distinguished.

detected by Δm = 16 Da, could be observed. (For details, see Table S4 in the Supporting Information.) The sample with the lowest ND was also found nitrosylated (Δm = 29 Da). Shifts in nominal masses were attributed to the modifications based on literature data.[31]

Bet v 1 contains a hydrophobic cavity[28,32] in which the peptide containing Y81 and Y83 is located (Figure 1b). Attraction of the hydrophobic nitrating agent TNM as well as the stabilization of tyrosyl radicals by hydrophobic environments[33] may explain why the preferred nitration site is Y83, which is also a key residue for the binding specificity of the hydrophobic pocket.[28] Lipidic plant mediators and amphipilic compounds such as steroids, cytokinins, and flavonids act as ligands for the hydrophobic cavity, suggesting a transport or storage function of Bet v 1.[28,34] Recently, a glycosylated flavonol (quercetin-3*O*-glycosid, Q3OS) has been shown to be a natural ligand of Bet v 1.[35] It is yet to be studied if a nitration of Y83 (or Y81) might alter the binding specificity of the hydrophobic pocket or its binding capacity.

Nitration with $ONOO^-$

The nitration of Bet v 1 with $ONOO^-$ in aqueous solution resulted in the NDs shown in Figure 3a. Again, total NDs were determined by AAA, HPLC−DAD, and LC−MS/MS and are plotted as a function of the molar ratio of $ONOO^-$ over tyrosine ($ONOO^-$/Y). Results of AAA and HPLC-DAD were consistent, whereas HPLC−MS/MS analysis showed slightly lower NDs. This may be due to unselective degradation of Bet v 1 during the reaction with $ONOO^-$ prior to protein digestion (see discussion later). The $ONOO^-$ reaction was studied by varying reaction time, reaction temperature, and the addition of a chelator (DTPA) to prevent the reaction of $ONOO^-$ with metal ions, such as iron or copper.[14] Higher NDs were observed for short reaction times (15 min), low temperatures (4 °C), and with the addition of DTPA (Table S1 in the Supporting Information). The ND_{max} did not exceed 0.5, which is ~70% of the ND_{max} for TNM. Modified sites with a level of modification <2% or with a not calculable level of modification are not shown in Figure 3. (For details, see Table S5 in the Supporting Information.) $ONOO^-$/Y molar ratios are approximate values due to the fast decomposition of $ONOO^-$.

ND_Y was found to vary strongly under the different reaction conditions, indicating a complex mixture of reaction products. (For details, see Table S5 in the Supporting Information.) For

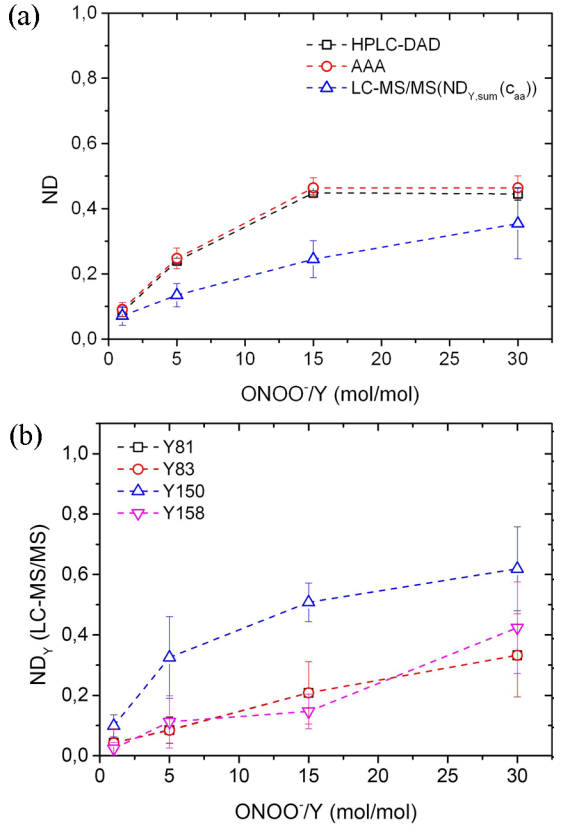

Figure 3. Nitration degrees for dissolved Bet v 1 nitrated by peroxynitrite ($ONOO^-$) in aqueous solution plotted against the molar ratio of nitrating agent and tyrosine residues ($ONOO^-$/Y): (a) total nitration degrees (ND) determined by AAA, HPLC-DAD, and LC−MS/MS and (b) site-specific nitration degrees (ND_Y) determined by LC−MS/MS. Data points and error bars represent the arithmetic mean values and standard errors of eight experiments.

reasons of simplicity, HPLC−MS/MS results were averaged over all experimental conditions, as illustrated in Figure 3b (arithmetic mean ± standard error of the mean (SEM), n = 8). The observed nitration pattern differed from that of TNM-nitrated Bet v 1, especially with regard to the peptide YNYSVIEGGPIGDTLEK, containing Y81 and Y83. The most efficiently nitrated tyrosine residue was Y150 located at the

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peptide AVESYLLAHSDAYN, that is, for all reaction conditions. Four to five of seven tyrosine residues could be identified with an amino acid sequence coverage of (68.2 ± 2.9)% (arithmetic mean ± standard deviation). Tyrosine residues Y5 and Y120 could not be detected in any of the runs, and Y66 could not be quantified reliably because the measured modified peptide (ISFPEGFPFKYVK, one missed cleavage, see Table 1) could not be compared with an unmodified peptide with the same sequence. The nitration potentially induced a missed cleavage for this peptide during digestion. However, some Y66 was found in hydroxylated (+ OH) and in nitrated form (+ NO_2). Hydroxylation of tyrosine and oxidation of methionine were also detected to a minor extent. (For details, see Table S5 in the Supporting Information.)

Intact protein analysis by HPLC–HR-MS indicated the addition of nitro-, nitroso-, and hydroxyl-groups to the protein as well as mixtures of the three modifications. (For details, see Table S6 in the Supporting Information.) Table S7 in the Supporting Information shows the relative abundances of unmodified (monoisotopic mass 17429 Da), oxidized, nitrated, degraded (fragments with molecular masses below 17 kDa), and other Bet v 1 signals in the mass spectra, comparing the reactions at 4 °C and at room temperature (~22 °C). Protein degradation was very prominent for $ONOO^-/Y = 30$ at room temperature (~85%), whereas at 4 °C, the other protein signals (>17.5 kDa) were the most relevant. These other signals were attributed to more complex reaction products with masses between the one of unmodified Bet v 1 and twice its molecular weight (aggregation products of degraded fragments, multiple combinations of different modifications).

For $ONOO^-$ nitration, Y150 was found to be a preferred nitration site of Bet v 1, which can be tentatively explained by the high solvent accessibility of Y150 (~25%).[32] This indicates a facilitated reaction of $ONOO^-$ with solvent-exposed tyrosine residues even at low concentrations. In addition, the negatively charged $ONOO^-$ is not expected to enter the hydrophobic cavity similarly to sulfate ions, which stay at the protein surface.[28] The tyrosine residue Y150 is located in the C-terminal region of Bet v 1,[36] which is supposed to be important for epitope binding[37] and is also part of a T-cell epitope and might thus be particularly relevant for changes in the allergenic potential of the protein.[3,4,36]

Nitration with O_3/NO_2

Nitration of Bet v 1 with O_3/NO_2 was performed in aqueous solution and heterogeneously via exposure of filter samples to gaseous O_3/NO_2 at different levels of RH, and the results are summarized in Table S2 in the Supporting Information and illustrated in Figure 4a.

The NDs observed upon exposure of filter samples did not exceed 2–4% at 45–92% RH but increased to ~6% under condensing conditions (>98% RH). In aqueous solution, the ND reached ~22%. Accordingly, the nitration rates for the filter samples of (semi)solid protein (ND ≈ 2% per day) were about one order of magnitude lower than that for the aqueous protein solution (ND ≈ 20% per day). This can be explained by a decrease in viscosity and an increase in diffusivity going from (semi)solid protein on filter to an aqueous solution[38−40] and described well by the kinetic multilayer model of aerosol surface and bulk chemistry,[41] as detailed in the Supporting Information (Section S4).

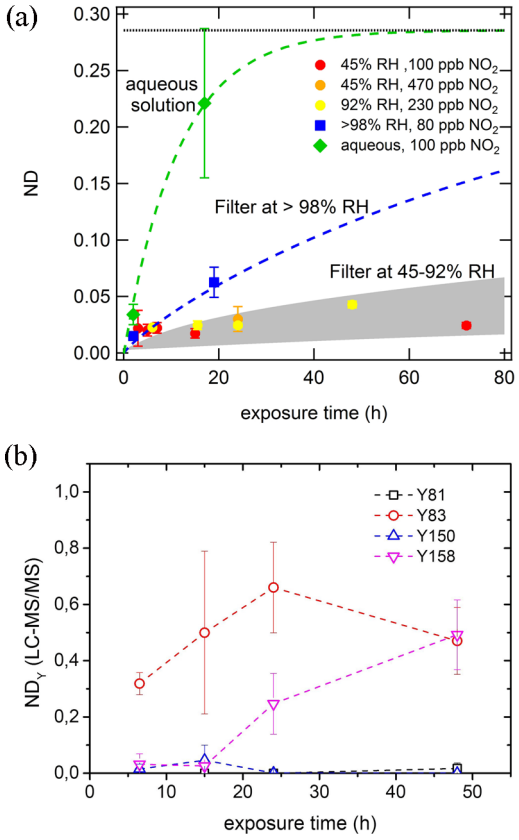

Figure 4. Nitration degrees for Bet v 1 exposed to ozone and nitrogen dioxide (O_3/NO_2) plotted against exposure time: (a) Total nitration degrees (ND) determined by HPLC–DAD for different types of samples and reaction conditions; data points and error bars represent the arithmetic mean values and standard errors of four experiments with filter samples and two experiments with liquid samples, respectively. The dashed lines and the gray shaded area show the results of kinetic model simulations, as indicated in the Figure and detailed in the Supporting Information (Section S4). The black dotted line represents the maximum ND for Bet v 1 based on the assumptions used in the model simulations. (b) Site-specific nitration degrees (ND_Y) determined by LC–MS/MS for filter samples exposed to 230 ppb of O_3/NO_2 at a relative humidity of 92%.

Intact protein analysis by HPLC–HR-MS of experiments exposing Bet v 1 on filters to gaseous O_3/NO_2 showed that in contrast with the ND the relative abundance of oxidized protein decreased with increasing RH. (See the Supporting Information Section S5.)

Analysis of the tryptic peptides indicated Y83 and Y158 to be the preferred sites for the nitration of Bet v 1 on filters for different reaction times (Figure 4b). For Y83, we observed a slight decrease at reaction times up to 48 h. This might be due to enhanced degradation and will be studied in more detail in follow-up studies. In addition, we observed high levels of oxidation at Y158 for short reaction times (for details, see Table S8 in the Supporting Information), supporting the assumption of ROIs to be involved in the first step of the nitration reaction.[22]

The nitration pattern for the heterogeneous reaction, that is, preference of Y158 and Y83 or Y81 (Table 2), was similar to the pattern observed for nitration by O_3/NO_2 in aqueous solution. This suggests similar reaction pathways for both types of reactions. Y158 is located at the C-terminus of the Bet v 1 molecule within a coil structure motif. Because coil structures

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belong to the most flexible parts of a protein, Y158 might have an increased probability for heterogeneous nitration because hydration dynamics occur fastest in lower-order structures.[42] Y83 and Y81 are located at the hydrophobic cavity and might be a preferred site due to a stabilization of tyrosyl radicals in hydrophobic environments and because most of the reactive nitrogen compounds are hydrophobic gases.[33]

4. CONCLUSIONS

In this study, we analyzed the reaction products of the birch pollen allergen Bet v 1 nitrated with the standard laboratory reagent for protein nitration (TNM) and two naturally occurring nitrating reagents, that is, $ONOO^-$ mimicking inflammation and oxidative/nitrosative stress and O_3/NO_2 mimicking air pollution effects. The results of this study show that both the efficiency and specificity of the protein nitration depend on the nitrating agent and the reaction conditions. TNM was found to be the most efficient nitrating reagent, yielding ND values up to 70%. $ONOO^-$ and O_3/NO_2 yielded ND values up to ~50 and ~20%, respectively, with substantial amounts of side products from protein oxidation and degradation. Nitration rates were about one order of magnitude higher for aqueous protein solutions (~20% per day) than for solid or semisolid protein samples (~2% per day). Thus, the allergenic potential of pollen and air particulate matter might be particularly enhanced under humid summer smog conditions, for example, in polluted tropical megacity regions.[43] The preferred reaction sites include tyrosine residues with high solvent accessibility or within a hydrophobic environment. Modification of the binding specificity of the hydrophobic pocket of Bet v 1 by nitration of Y83 and modification of the C-terminal helix crucial for epitope binding by nitration of Y150 and/or Y158 might be particularly relevant for changes in the allergenic potential of the protein.[3,4,28,36]

■ ASSOCIATED CONTENT

Supporting Information

Experimental setups used for the exposure of Bet v 1 to O_3/NO_2 mixtures. Exemplary MS/MS spectra of tryptic Bet v 1 peptides. Experimental conditions for $ONOO^-$-nitrated and O_3/NO_2-exposed Bet v 1 samples. MS intensities of nitrated and unmodified synthetic peptides of Bet v 1. Masses of Bet v 1 nitrated with TNM to different degrees detected with LC−HR-MS. LC−MS/MS analysis of Bet v 1 nitrated by $ONOO^-$. Masses of Bet v 1 nitrated with different amounts of $ONOO^-$ detected with LC−HR-MS. Intact protein analysis by HPLC−HR-MS Bet v 1. Reaction of Bet v 1 on filters and in aqueous solution with O_3/NO_2 at different reaction times. Triplicate LC−MS/MS analysis of Bet v 1. Protein digestion and HPLC−MS/MS analysis. HPLC−HR-MS analysis of intact Bet v 1.0101. Experimental details for the nitration of Bet v 1 using O_3/NO_2 mixtures. Kinetic model simulations. Detailed discussion of the results for heterogeneous and homogeneous nitration of Bet v 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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