

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

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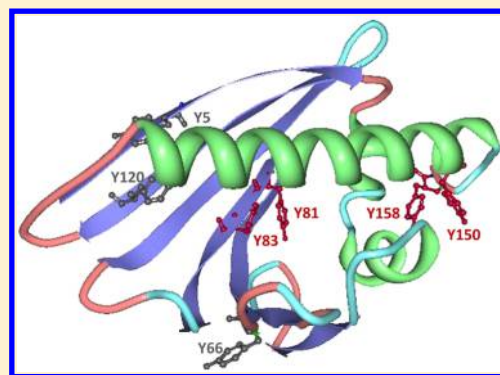
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Supporting Information

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, O₃/NO₂). 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 O₃/NO₂. 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 O₃/NO₂ 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 allergens³ 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 (O₃).^{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 (O₂⁻) can form

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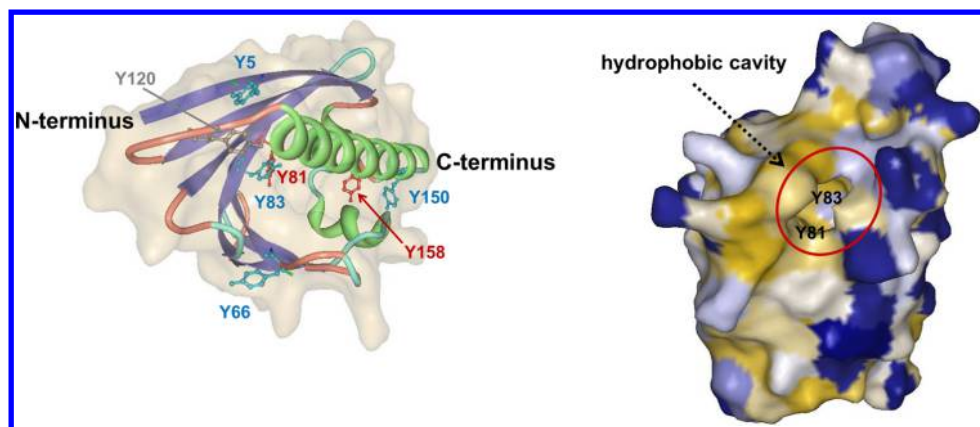


Figure 1. Position of tyrosine residues in the 3-D structure of crystallized unmodified Bet v 1.0101 (PDB accession code: 4A88)²⁸ created with the PDB protein workshop 3.9.⁴⁴ (left) Tyr residues indicated in blue (Y5, Y66, Y83, and Y150) have approximate values of solvent exposure of >20%, the red-indicated 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 peroxytrinitrite (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, NO₂, and carbonate radicals CO₃⁻) attack the aromatic ring, leading to the formation of a Tyr radical. In a second step, the Tyr radical combines with NO₂ 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 (NO₂) and ozone (O₃). Laboratory and field studies showed that proteins were efficiently nitrated upon exposure to gas mixtures of NO₂ and O₃ or polluted urban air (summer smog).²¹ The heterogeneous reaction of the protein with the gaseous reactants O₃ and NO₂ 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 O₃. The conclusion of these studies was that the nitration reaction of proteins with O₃ and NO₂ proceeds through long-lived reactive oxygen intermediates (ROIs).²³ The protein first reacts with O₃ and forms a ROI, most likely Tyr radicals, which can persist over extended periods of time. In a second step, the ROI reacts with NO₂, resulting in the formation of 3-nitrotyrosine residues. This two-step mechanism, including an oxidation step and an NO₂ addition step, is comparable to the nitration mechanism of ONOO⁻. In the case of ONOO⁻ nitration, the oxidants for the first step are CO₃⁻ radicals and oxo-metal complexes or, to a lesser extent, OH and NO₂ radicals, while for the heterogeneous reaction with the gaseous components O₃ is the oxidizing agent. The second step in both cases is the addition of either exogenous or endogenous NO₂. 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 O₃ in combination with NO₂ 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 described²⁸ 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 PO₄ buffer with pH 7.4.

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 Na₂HPO₄, 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 rebuffed in a 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) to a final allergen concentration of 1.0 mg mL⁻¹, as previously described.²⁴ Sodium peroxyxynitrite (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 Peroxyxynitrite is stable under alkaline aqueous solutions, but it decomposes at a lower pH, where the peroxyxynitrite anion is protonated and forms peroxyxynitrous acid (ONOOH), which can form hydroxyl radicals (OH) and nitrogen dioxide radicals (NO₂).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 O₃/NO₂

Bet v 1 was exposed to O₃/NO₂ 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 O₃/NO₂ mixtures at different RH. Additionally, the homogeneous reaction of the dissolved protein and the reactants was studied by bubbling O₃/NO₂ 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 described²⁴ 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 YNYVIEGGPGDT-LEK (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·H₂O for modified Y81) and *m/z* 321.1 (*b*₂ for nitrated Y81 and *b*₇⁺²·H₂O 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.

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		VAPQAISVENIEGNGGPGTIK	16.44	713.3744	2138.1088
33–55		VAPQAISVENIEGNGGPGTIKK	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	ISFPEGFPFKYVK	21.90	535.2755	1558.83
69–80		DRVDEVDHTNFK	11.76	369.4242	1474.6921
71–80		VDEVHTNFK	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	AVESYLLAHSDAYN	17.69	799.3589	1552.7278
146–159	158	AVESYLLAHSDAYN	17.76	533.2367	1552.7278
146–159	150, 158	AVESYLLAHSDAYN	19.13	821.8481	1552.7278

^aAmino 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 O₃/NO₂, and heterogeneously via exposure of Bet v 1 filter samples to gaseous O₃/NO₂ 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 (GVFNYYETTSVIPAAAR), 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

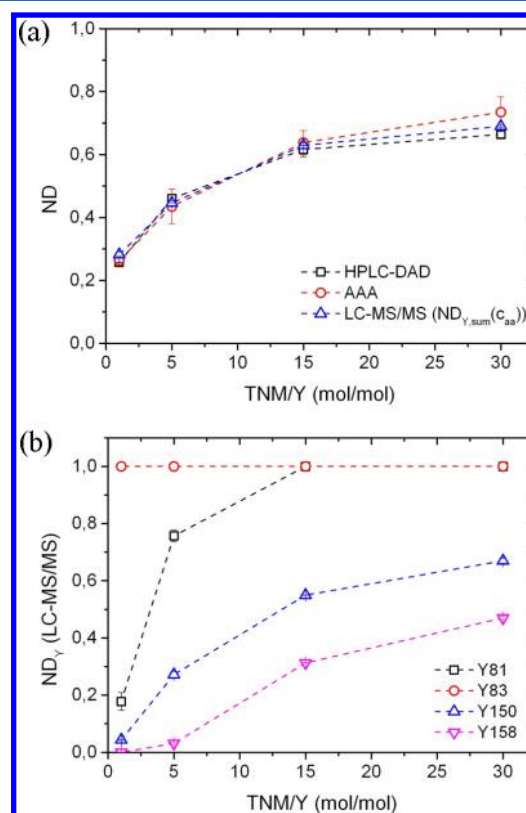


Figure 2. Nitration degrees for dissolved Bet v 1 nitrated by tetranitromethane (TNM) in solution plotted against the molar ratio of nitrating agent and tyrosine residues (TNM/Y): (a) total nitration degrees (ND) determined by AAA, HPLC–DAD, and LC–MS/MS and (b) site-specific nitration degrees (ND_Y)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

phenylalanine or the oxidation of methionine was not observed in the HPLC–MS/MS analysis.

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

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 O₃/NO₂ (ND = 20%) and for the Heterogeneous Reaction with O₃/NO₂ (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

^aThe 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. ^bReaction in aqueous solution with TNM/Y = 1 with a reaction time of 60 min, *n* = 4 (analytical replicates). ^cReaction 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). ^dReaction 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). ^eHeterogeneous reaction of protein on filter with 230 ppb O₃ and 230 ppb NO₂ with a reaction time of 48 h (experiment 16, Table S2 in the Supporting Information), *n* = 4 (experimental replicates). ^fY81 and Y83 not clearly distinguished.

detected by $\Delta m = 16$ Da, could be observed. (For details, see Table S4 in the Supporting Information.) The sample with the lowest ND was also found nitrosylated ($\Delta m = 29$ 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 environments³³ 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 flavonoids 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

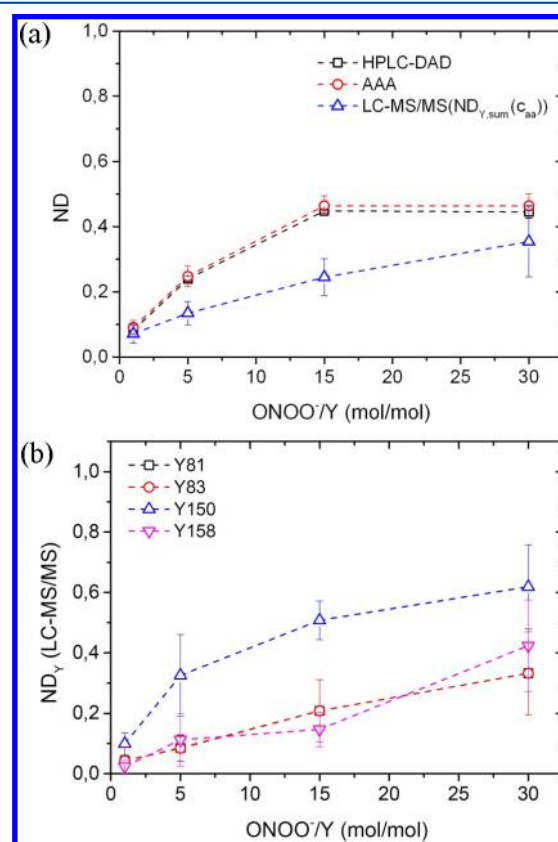


Figure 3. Nitration degrees for dissolved Bet v 1 nitrated by peroxynitrite (ONOO[−]) in aqueous solution plotted against the molar ratio of nitrating agent and tyrosine residues (ONOO[−]/Y): (a) total nitration degrees (ND) determined by AAA, HPLC–DAD, and LC–MS/MS and (b) site-specific nitration degrees (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

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 $(68.2 \pm 2.9)\%$ (arithmetic mean $\pm$ 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 (+ NO₂). 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 ($\sim 85\%$), 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 ($\sim 25\%$).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 binding³⁷ 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 O₃/NO₂

Nitration of Bet v 1 with O₃/NO₂ was performed in aqueous solution and heterogeneously via exposure of filter samples to gaseous O₃/NO₂ 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 $\sim 6\%$ under condensing conditions ($>98\%$ RH). In aqueous solution, the ND reached $\sim 22\%$. Accordingly, the nitration rates for the filter samples of (semi)solid protein (ND $\approx 2\%$ per day) were about one order of magnitude lower than that for the aqueous protein solution (ND $\approx 20\%$ 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).

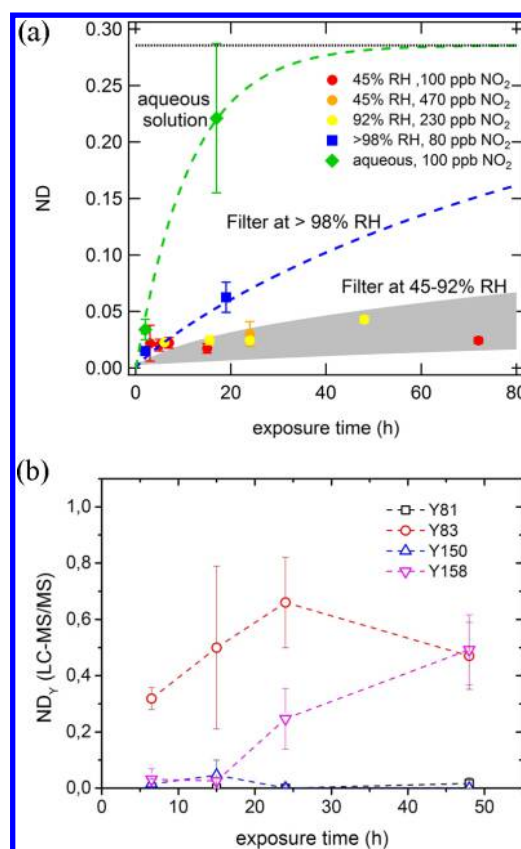


Figure 4. Nitration degrees for Bet v 1 exposed to ozone and nitrogen dioxide (O₃/NO₂) plotted against exposure time: (a) Total nitration degrees (ND) determined by HPLC–DAD for different types of samples and reaction conditions; data points and error bars represent the arithmetic mean values and standard 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 O₃/NO₂ at a relative humidity of 92%.

Intact protein analysis by HPLC–HR-MS of experiments exposing Bet v 1 on filters to gaseous O₃/NO₂ 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 O₃/NO₂ 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

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 O₃/NO₂ 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 O₃/NO₂ 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 O₃/NO₂ mixtures. Exemplary MS/MS spectra of tryptic Bet v 1 peptides. Experimental conditions for ONOO[−]-nitrated and O₃/NO₂-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 O₃/NO₂ 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 O₃/NO₂ 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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Supplementary Information:

“Nitration of the birch pollen allergen Bet v 1.0101: efficiency and site-selectivity of liquid and gaseous nitrating agents”

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

SUPPLEMENTARY FIGURES

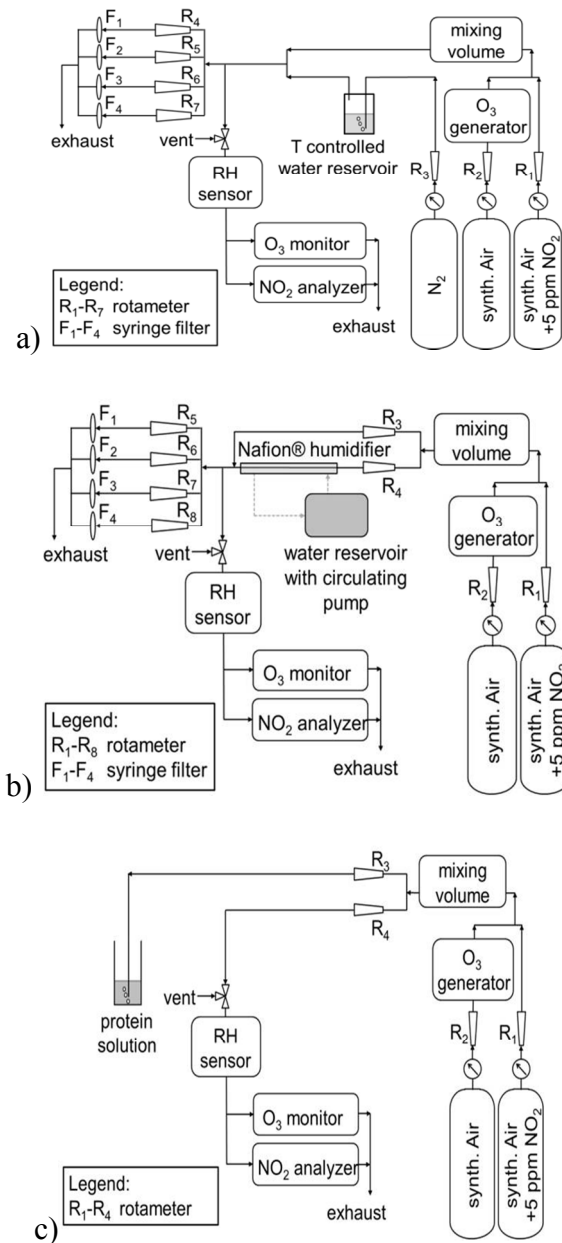


Figure S1. Experimental setups used for the exposure of Bet v 1 to O₃/NO₂ mixtures. a) setup 1 used for experiments 1-10 (Table S2), b) setup 2 used for experiments 11-15 (Table S2), and c) setup 3 used for experiments 16-17. For detailed information on experimental procedures see supplementary information section S3.

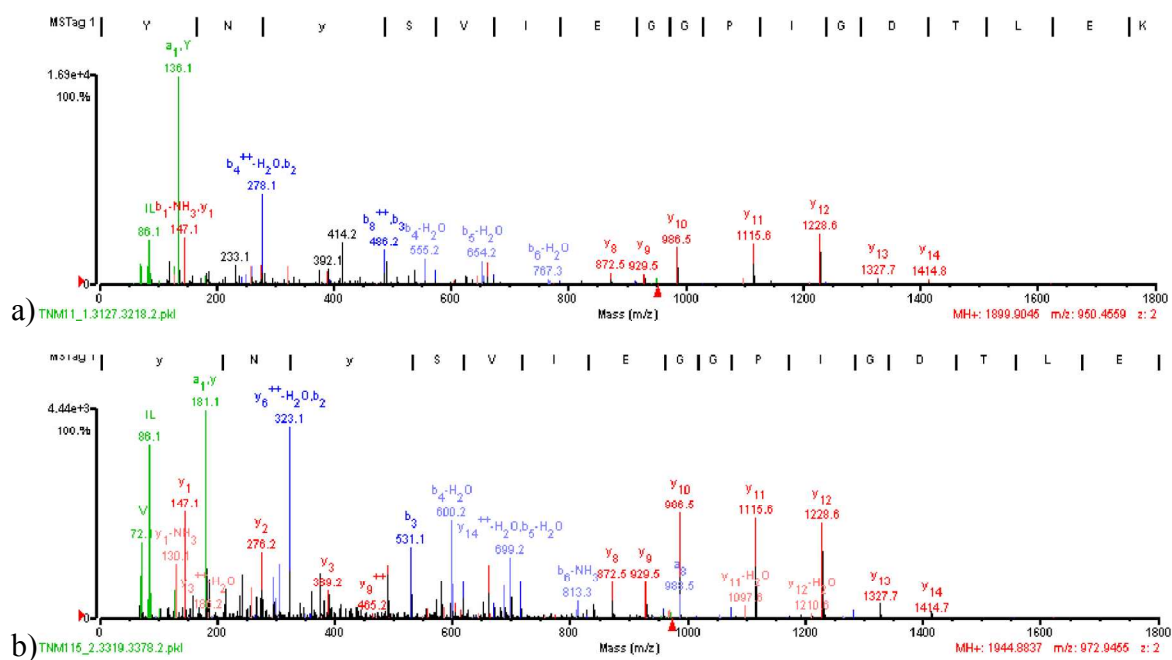


Figure S2. Exemplary MS/MS spectra of tryptic Bet v 1 peptide Y₈₁NY₈₃SVIEGGPIGDTLEK nitrated by TNM: a) nitrated Y₈₃ (score: 15.59, % SPI: 82.8 and MH⁺ error: 3.9 ppm) and b) nitrated Y₈₁ and Y₈₃ (score: 19.12, % SPI: 90.2, MH⁺ error: 0.8 ppm).

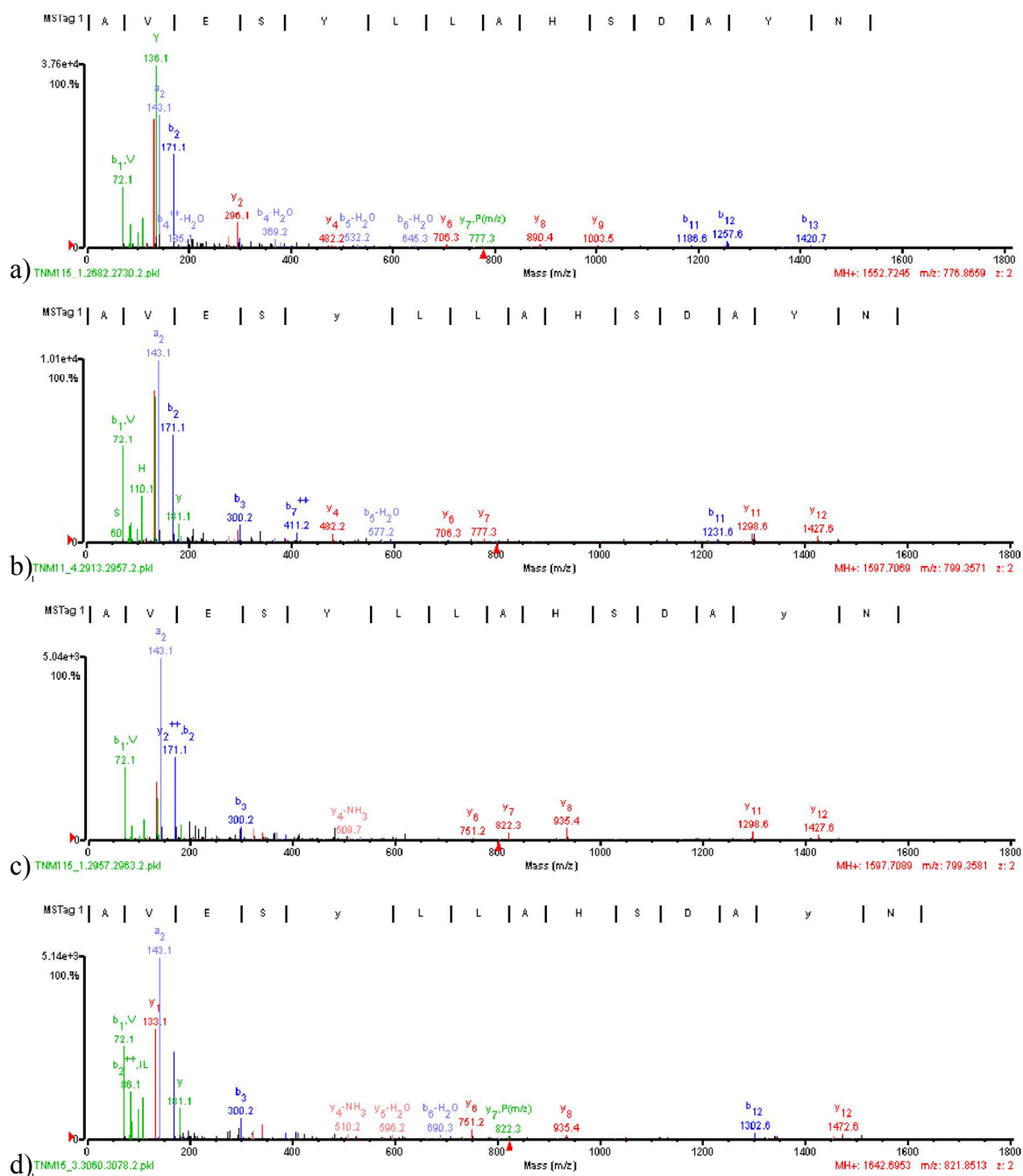


Figure S3. Exemplary MS/MS spectra of tryptic Bet v 1 peptide AVESAVESY150LLAHSDAY158N nitrated by TNM: a) unmodified Y150 and Y158 (score: 14.68, % SPI: 89.9 and MH⁺ error: -1.5 ppm), b) nitrated Y150 (score: 18.39, % SPI: 95.2, MH⁺ error: -3.7 ppm), c) nitrated Y158 (score: 16.85, % SPI: 93.4, MH⁺ error: -2.5 ppm), and d) nitrated Y150 and Y158 (score: 16.7, % SPI: 88.9, MH⁺ error: -1.6 ppm).

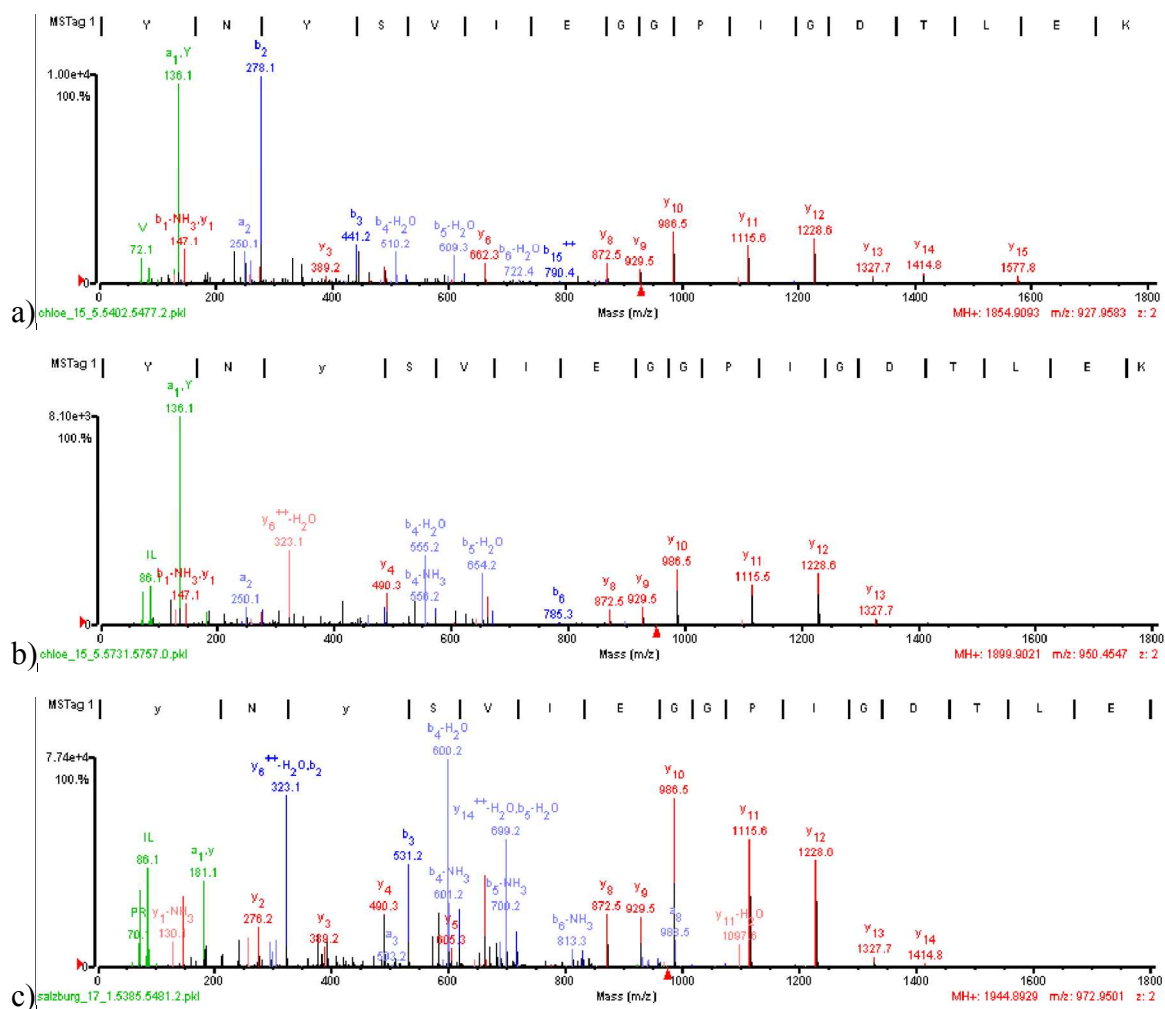


Figure S4. Exemplary MS/MS spectra of tryptic Bet v 1 peptide Y₈₁NY₈₃SVIEGGPIGDTLEK nitrated by ONOO⁻: a) unmodified Y₈₁ and Y₈₃ (score: 18.50, % SPI: 85.0 and MH⁺ error: -1.4 ppm), b) nitrated Y₈₁ or Y₈₃, which was not distinguishable due to the signal at m/z 323.1 (b₂ of nitrated Y₈₁ or y₆⁺⁺-H₂O of nitrated Y₈₃ (score: 15.39, % SPI: 85.3, MH⁺ error: 2.7 ppm), and c) nitrated Y₈₁ and Y₈₃ (score: 18.5, % SPI: 91.6, MH⁺ error: 5.5 ppm).

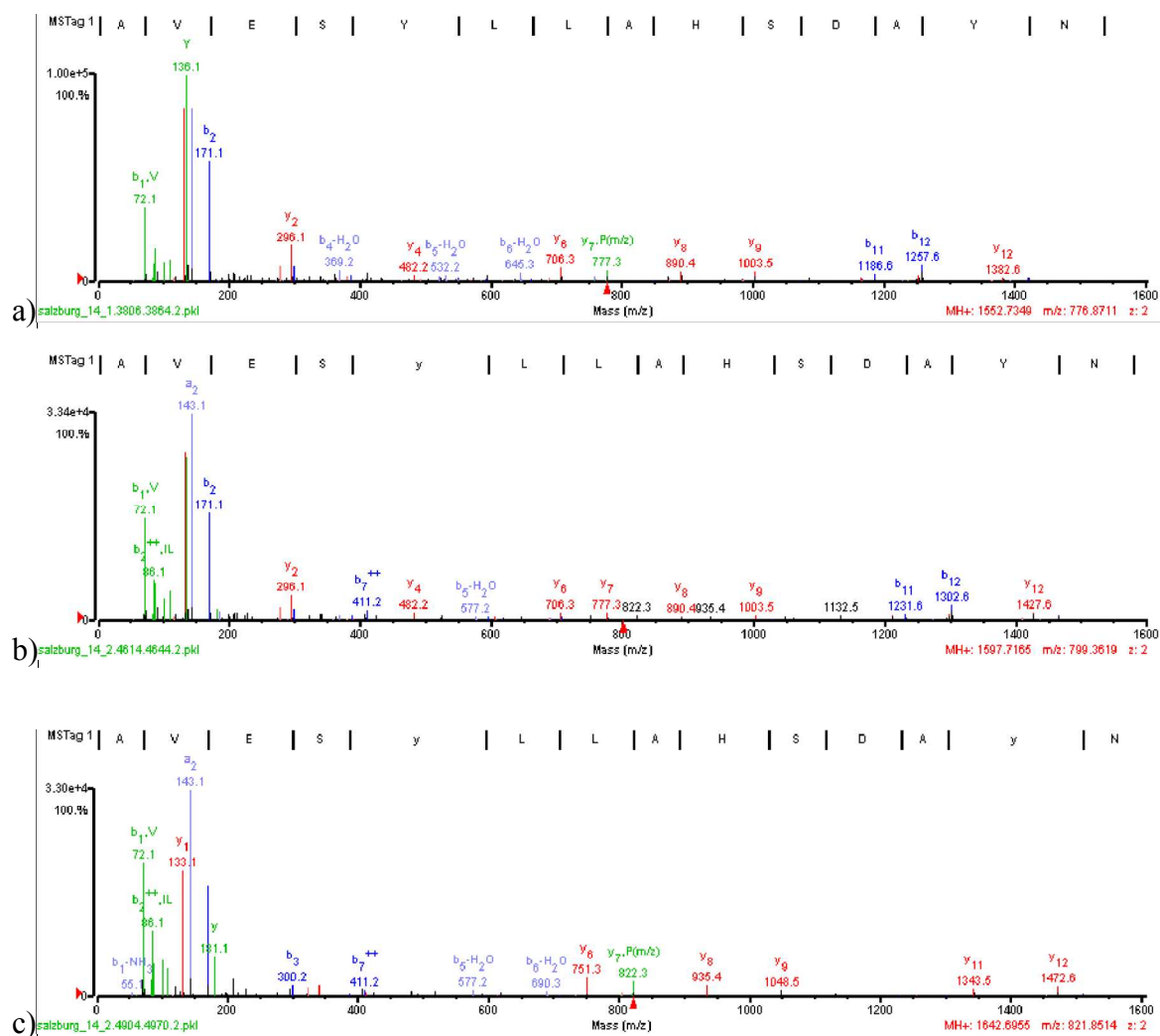


Figure S5. Exemplary MS/MS spectra of tryptic Bet v 1 peptide AVESAVESY₁₅₀LLAHSDAY₁₅₈N nitrated by ONOO⁻: a) unmodified Y150 and Y158 (score: 18.06, % SPI: 94.4 and MH⁺ error: 4.6 ppm), b) nitrated Y150 (score: 18.88, % SPI: 94.9, MH⁺ error: 2.3 ppm), and c) nitrated Y150 and Y158 (score: 17.82, % SPI: 92.4, MH⁺ error: -1.5 ppm).

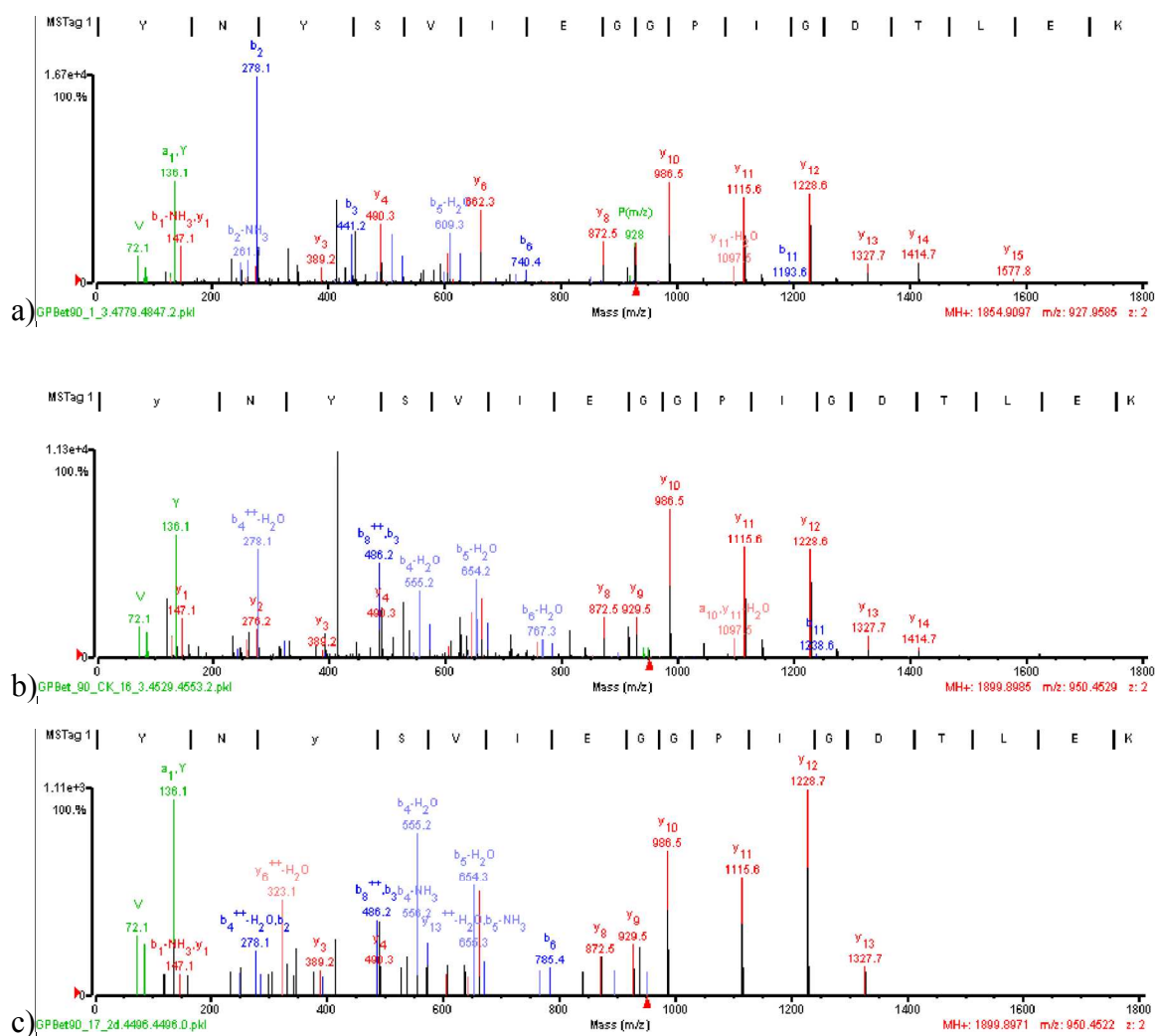


Figure S6. Exemplary MS/MS spectra of tryptic Bet v 1 peptide Y₈₁NY₈₃SVIEGGPIGDTLEK heterogeneously nitrated by O₃/NO₂: a) unmodified Y₈₁ and Y₈₃ (score: 17.26, % SPI: 82.0 and MH⁺ error: -3.4 ppm), b) nitrated Y₈₁ (score: 13.48, % SPI: 74.3, MH⁺ error: 0.8 ppm), and c) nitrated Y₈₃ (score: 13.34, % SPI: 79.3, MH⁺ error: 0.0 ppm).

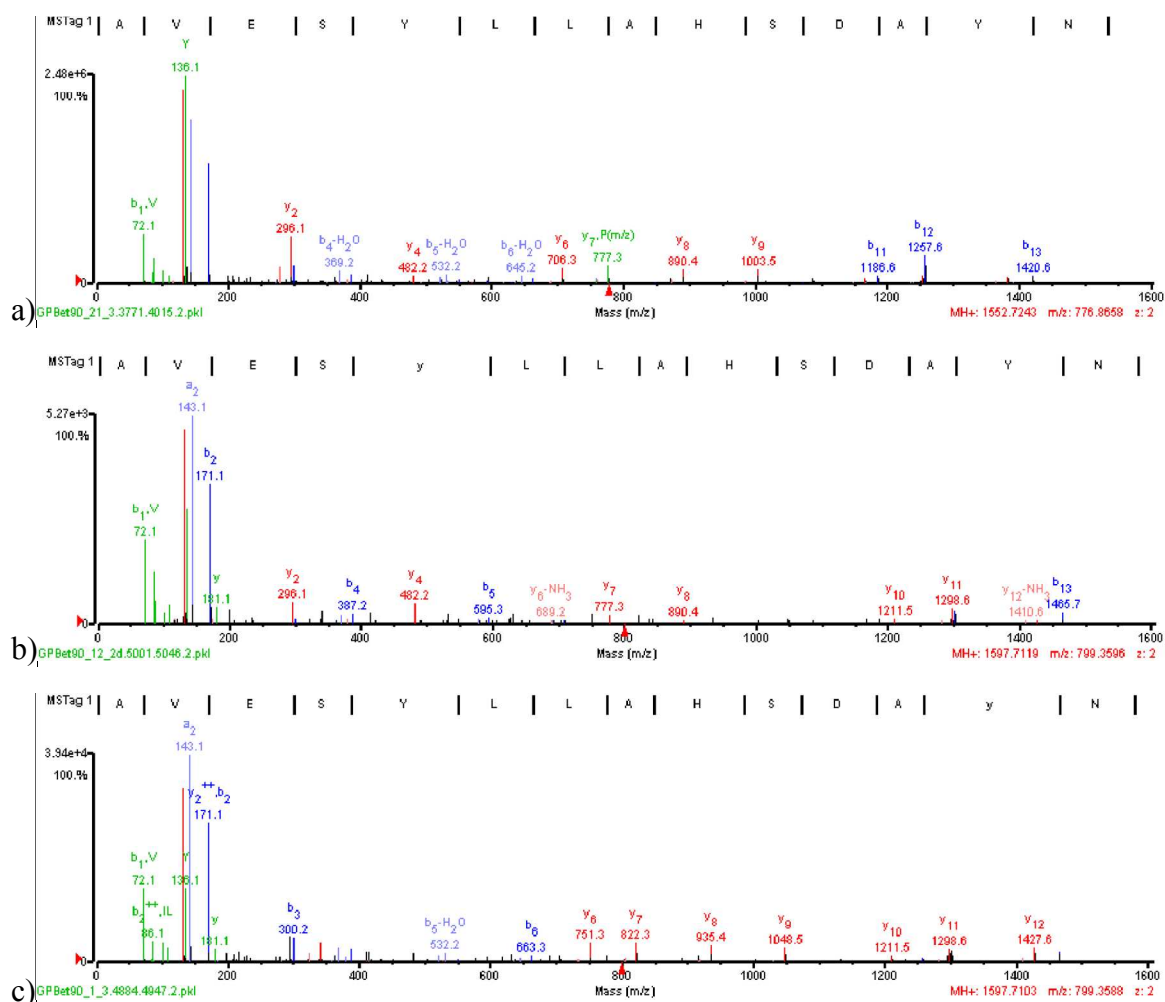
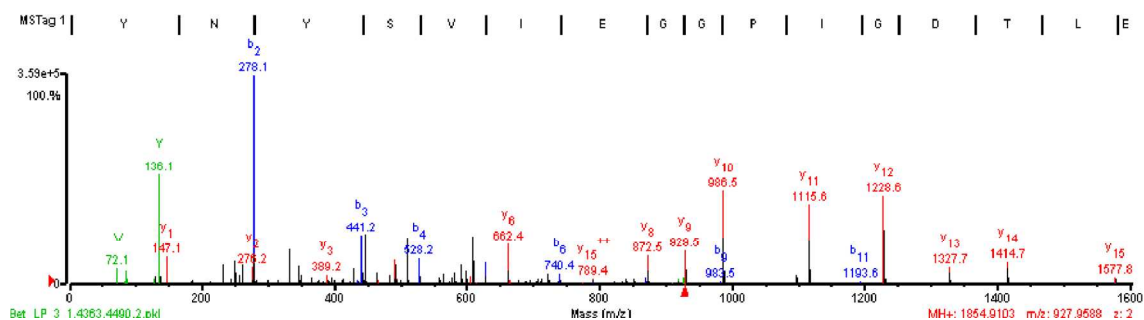
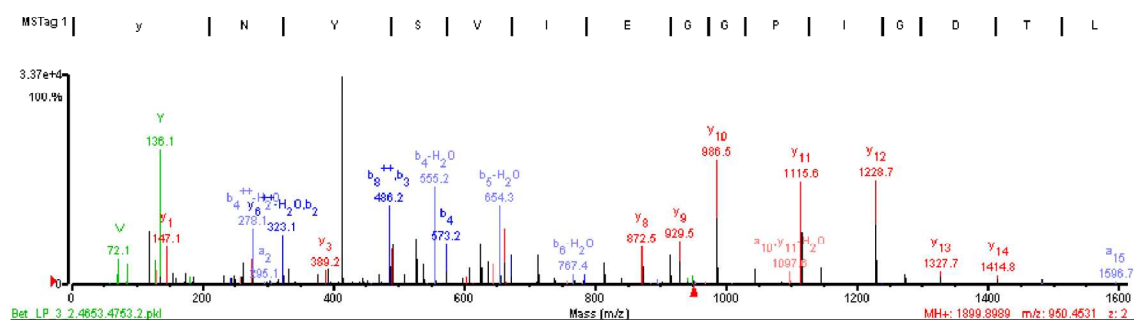


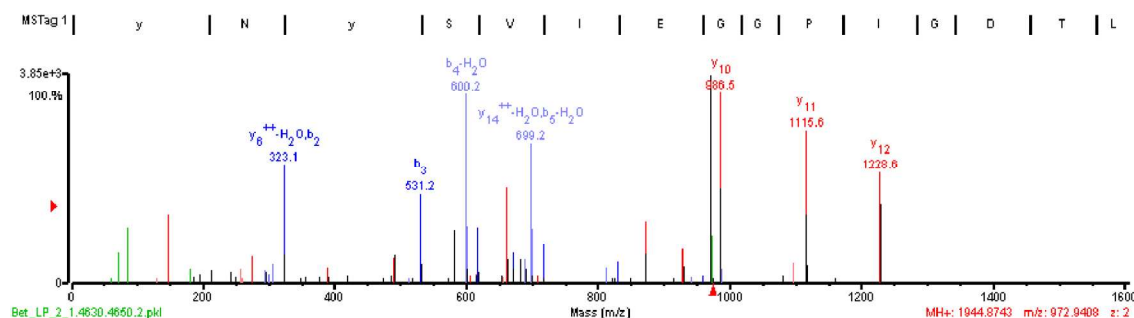
Figure S7. Exemplary MS/MS spectra of tryptic Bet v 1 peptide AVESAVESY₁₅₀LLAHSDAY₁₅₈N heterogeneously nitrated by O₃/NO₂: a) unmodified Y150 and Y158 (score: 18.65, % SPI: 96.4 and MH⁺ error: 0.2 ppm), b) nitrated Y150 (score: 13.73, % SPI: 85.1, MH⁺ error: -0.6 ppm), and c) nitrated Y158 (score: 21.13, % SPI: 95.8, MH⁺ error: 1.6 ppm).



a)



b)



c)

Figure S8. Exemplary MS/MS spectra of tryptic Bet v 1 peptide Y₈₁NY₈₃SVIEGGPIGDTLEK nitrated in aqueous solution by O₃/NO₂: a) unmodified Y81 and Y83 (score: 17.70, % SPI: 85.2 and MH⁺ error: 0.9 ppm), b) nitrated Y81 or Y83, which was not distinguishable due to the signal at m/z 278.1 (b₂ of unmodified Y81 or b₄⁺⁺-H₂O of nitrated Y81 (score: 13.92, % SPI: 73.9, MH⁺ error: 1 ppm), and c) nitrated Y81 and Y83 (score: 19.16, % SPI: 95.2, MH⁺ error: -3.5 ppm).

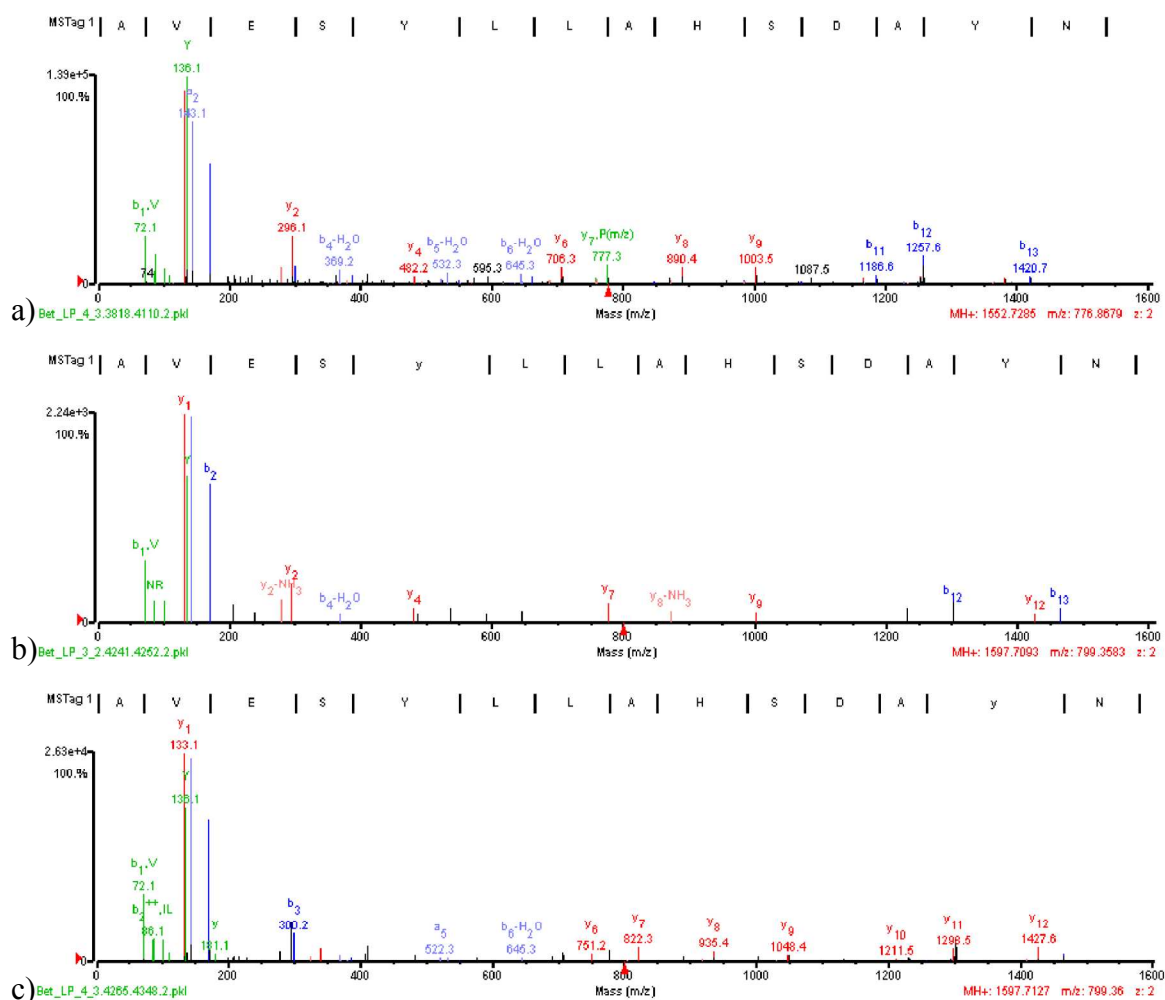


Figure S9. Exemplary MS/MS spectra of tryptic Bet v 1 peptide AVESAVESY₁₅₀LLAHSDAY₁₅₈N nitrated in aqueous solution by O₃/NO₂: a) unmodified Y150 and Y158 (score: 19.91, % SPI: 96.3 and MH⁺ error: -2.4 ppm), b) nitrated Y150 (score: 13.96, % SPI: 90.6, MH⁺ error: -2.2 ppm), and c) nitrated Y158 (score: 18.18, % SPI: 84.8, MH⁺ error: -0.1 ppm).

SUPPLEMENTARY TABLES

Table S1. Experimental conditions for ONOO⁻ nitrated Bet v 1. The total nitration degree is determined by LC-DAD and by AAA.

Exp. no.	ONOO ⁻ /Y (mol mol ⁻¹)	Temp. (°C)	Time (min)	DTPA	ND (LC-DAD) (mean ± SD, n = 3)	ND (AAA) (mean ± SD, n = 2)
1	30	4	15	-	0.580 ± 0.015	0.512 ± 0.075
2	15	4	15	-	0.455 ± 0.004	0.553 ± 0.018
3	5	4	15	-	0.269 ± 0.001	0.275 ± 0.038
4	1	4	15	-	0.099 ± 0.001	0.081 ± 0.011
5	30	4	100	-	0.462 ± 0.005	0.458 ± 0.077
6	15	4	100	-	0.504 ± 0.005	0.446 ± 0.110
7	5	4	100	-	0.256 ± 0.007	0.301 ± 0.041
8	1	4	100	-	0.098 ± 0.001	0
9	30	22	15	-	0.489 ± 0.008	0.327 ± 0.019
10	15	22	15	-	0.492 ± 0.015	0.310 ± 0.029
11	5	22	15	-	0.263 ± 0.003	0.164 ± 0.032
12	1	22	15	-	0.025 ± 0.001	0.051 ± 0.072
13	30	22	100	-	0.413 ± 0.002	0.424 ± 0.052
14	15	22	100	-	0.481 ± 0.002	0.470 ± 0.086
15	5	22	100	-	0.234 ± 0.008	0.102 ± 0.144
16	1	22	100	-	0.123 ± 0.008	0.119 ± 0.072
17	30	4	15	+	0.539 ± 0.009	0.640 ± 0.025
18	15	4	15	+	0.465 ± 0.002	0.541 ± 0.003
19	5	4	15	+	0.265 ± 0.003	0.322 ± 0.005
20	1	4	15	+	0.024 ± 0.002	0.089 ± 0.005
21	30	4	100	+	0.437 ± 0.009	0.429 ± 0.017
22	15	4	100	+	0.499 ± 0.002	0.521 ± 0.058
23	5	4	100	+	0.223 ± 0.002	0.287 ± 0.022
24	1	4	100	+	0.101 ± 0.001	0.178 ± 0.106
25	30	22	15	+	0.484 ± 0.020	0.505 ± 0.100
26	15	22	15	+	0.480 ± 0.001	0.450 ± 0.118
27	5	22	15	+	0.215 ± 0.005	0.179 ± 0.017
28	1	22	15	+	0.089 ± 0.001	0.062 ± 0.011
29	30	22	100	+	0.393 ± 0.015	0.416 ± 0.087
30	15	22	100	+	0.473 ± 0.007	0.421 ± 0.027
31	5	22	100	+	0.292 ± 0.011	0.350 ± 0.025
32	1	22	100	+	0.118 ± 0.020	0.147 ± 0.015

Table S2. List of experimental conditions for O₃/NO₂-exposed Bet v 1 samples.

Exp. no.	Time (h)	RH (%)	$m_{\text{Filter}} (\mu\text{g})/$ $c_{\text{Solution}} (\text{mg mL}^{-1})^{\text{a}}$	O ₃ (ppb)	NO ₂ (ppb)	ND (LC-DAD)	ND (AAA)
1	3	47	290	100	100	0.022 ± 0.016	0.000
2	7	47	290	100	100	0.022 ± 0.001	0.059
3	15	47	290	100	100	0.016 ± 0.004	0.037
4	72	47	290	100	100	0.024 ± 0.003	0.000
5	5	47	290	320	320	0.020 ± 0.005	0.042
6	24	47	290	470	470	0.034 ± 0.012	-
7	1	>98 ^b	162	36	64	0.000 ± 0.000	0.10
8	16	>98 ^b	162	180	180	0.100 ± 0.001	-
9	19	>98 ^b	162	10	80	0.068 ± 0.013	0.051
10	2	>98 ^b	162	10	80	0.010 ± 0.003	-
11	48	92	130	230	230	0.036 ± 0.011	-
12	24	92	130	230	230	0.024 ± 0.002	-
13	15	92	130	235	230	0.025 ± 0.004	-
14	6.5	92	130	230	233	0.023 ± 0.001	-
15	16	98	130	230	230	0.028 ± 0.002	-
16	2	liquid	1.6	100	100	0.034 ± 0.009	-
17	17	liquid	0.16	120	120	0.221 ± 0.066	-

^a m_{Filter} = mass of protein loaded onto the filter, c_{Solution} = concentration of protein dissolved in water for liquid-phase experiment

^b condensation observed in tubing and on filters

Table S3. MS intensities of nitrated and unmodified synthetic peptides of Bet v 1. The area values are normalized due to the concentration difference in the BCA assay. The mean difference in MS intensities is 0.17 ± 0.12 (arithmetic mean $\pm$ standard deviation, $n = 3$). Marked tyrosine residues (Y) are nitrated.

Peptide	RT (min)	Area (*10 ⁷)	Area (%)	m/z	Conc. (mgmL ⁻¹)	Dil. Fac.	Norm. Area (%)	Δ Area (%)
NYETETTSVIPAAPL	11.48	3.40	100.00	832.9242	0.98	1.00	100.00	0.00
NYETETTSVIPAAPL	12.07	2.62	77.16	855.4135	0.84	1.17	90.02	9.98
SILKISNKYHTK	10.43	3.42	44.20	358.7996	0.21	2.95	130.50	30.50
SILKISNKYHTK	11.19	7.73	100.00	369.9561	0.62	1.00	100.00	0.00
TLLRAVESYLLAHS	14.25	6.83	100.00	562.3138	1.09	1.00	100.00	0.00
TLLRAVESYLLAHS	16.48	3.72	54.53	578.2957	0.66	1.65	90.06	9.94

Table S4. Masses of Bet v 1 nitrated with TNM to different degrees detected with LC-HR-MS.

Masses	30/1		15/1		5/1		1/1		modification
	%	SD	%	SD	%	SD	%	SD	
17457.9350	0	0	0	0	0.30	0.26	2.27	2.30	+ 29
17472.9238	0	0	0	0	0.89	1.55	25.63	3.57	+ 1 NO ₂ - 1
17488.9750	0	0	0	0	0	0	11.32	0.74	+ 1 NO ₂ + 15
17518.8806	0.46	0.65	1.02	1.30	13.44	5.12	29.06	2.07	+ 2 NO ₂
17533.9133	0	0	0	0	0	0	6.04	1.90	+ 2 NO ₂ + 15
17563.8894	2.02	2.50	7.93	1.83	23.91	2.28	11.02	2.72	+ 3 NO ₂ + 1
17608.8880	10.57	2.27	18.62	2.92	19.48	4.33	0.38	0.66	+ 4 NO ₂ - 1
17624.9492	5.41	2.85	5.55	5.07	0	0	0	0	+ 4 NO ₂ + 16
17652.9020	19.31	3.63	22.57	3.53	7.95	3.73	0	0	+ 5 NO ₂ - 1
17668.9372	7.56	0.67	2.71	4.69	0.75	1.29	0	0	+ 5 NO ₂ + 16
17697.8948	19.07	3.03	13.51	1.12	0.94	1.19	0	0	+ 6 NO ₂ - 1
17713.9220	4.72	4.09	2.57	2.23	0.07	0.13	0	0	+ 6 NO ₂ + 16
17742.8796	9.77	2.09	1.15	1.99	0.08	0.13	0	0	+ 7 NO ₂ - 1
17758.8914	4.29	1.46	0.07	0.13	0	0	0	0	+ 7 NO ₂ + 15

Table S5. LC-MS/MS analysis of Bet v 1 nitrated by ONOO⁻. The nitration and oxidation sites of three analytical replicates were determined and categorized. (+++) modified site is found in all three analytical replicates with high intensities, (++) modified site is found in two of three analytical replicates with high intensities or found in all three analytical replicates with low intensities, (+) modified site is found in one of three analytical replicates with high or low intensities or found in two of three analytical replicates with low intensities, and (-) modified site is not found in any of the runs. The mean intensity for the LC-MS/MS runs was $(5.04 \pm 0.92)10^6$ (arithmetic mean $\pm$ standard deviation). High intensity is defined as higher than 10% of the mean intensity ($\sim 10^5$ to 10^6) and low intensity is defined as lower than 1% of the mean intensity ($\sim 10^4$).

Reaction conditions				Y66 +NO ₂	Y66 +OH	Y81 +NO ₂	Y81 +OH	Y83 +NO ₂	Y83 +OH	M139 + O	Y150 +NO ₂	Y150 +OH	Y158 +NO ₂	Y158 +OH	
Without DTPA	4 °C	15 min	30/1	-	++	++	-	++	-	-	+++	+	+++	++	
			15/1	-	-	+++	-	+++	-	-	+++	-	++	-	
			5/1	-	-	+++	-	+++	-	+	+++	-	+++	-	
			1/1	-	-	-	-	-	-	-	+++	++	++	++	
		100 min	30/1	-	-	+++	-	+++	-	-	+++	+	+++	+	
			15/1	-	-	++	-	++	-	-	+++	-	++	-	
			5/1	-	-	-	+	-	+	-	+++	-	++	-	
			1/1	-	-	-	-	-	-	-	++	-	-	-	
	RT	15 min	30/1	-	-	+	++	+	++	-	+++	+	+++	+	
			15/1	-	-	++	-	++	-	-	+++	++	+++	++	
			5/1	-	-	+++	++	+++	++	-	+++	++	+++	++	
			1/1	-	-	-	-	-	-	-	+++	-	-	-	
		100 min	30/1	-	-	++	-	++	-	-	+++	+	+++	+	
			15/1	-	-	+++	-	+++	-	-	+++	+	+++	+	
			5/1	-	-	-	-	-	-	-	-	-	-	-	
			1/1	-	-	-	-	-	-	-	+++	-	-	-	
With DTPA	4 °C	15 min	30/1	-	+	+++	-	+++	-	-	+++	++	+++	++	
			15/1	-	-	+++	-	+++	-	-	+++	-	+++	-	
			5/1	-	-	+++	-	+++	-	-	+++	-	++	-	
			1/1	-	-	-	-	-	-	-	-	-	-	-	
		100 min	30/1	-	-	-	-	-	-	-	-	-	-	-	-
			15/1	-	-	+++	-	+++	-	+	+++	-	+++	-	
			5/1	-	-	-	-	+++	-	+	-	-	-	-	
			1/1	-	-	-	-	+	-	+	++	-	-	-	
	RT	15 min	30/1	-	-	-	-	+	-	+	+++	++	++	++	
			15/1	-	-	++	-	++	-	+	+++	-	+	-	
			5/1	-	-	++	-	++	-	+	+++	+	++	-	
			1/1	-	-	+	-	+	-	+	++	-	++	-	
		100 min	30/1	-	-	+++	-	+++	-	+++	+++	-	+++	-	
			15/1	+++	-	-	-	-	-	+++	+++	+++	+++	+++	
			5/1	-	-	-	-	-	-	++	+++	-	+++	-	
			1/1	+	-	-	-	-	-	-	+++	-	-	-	
Σ (+)				4	2	44	5	49	5	16	81	19	59	19	

Table S6. Masses of Bet v 1 nitrated with different amounts of ONOO⁻ (17 molar excess to 0.58 molar excess of ONOO⁻ over tyrosine) detected with LC-HR-MS. The experiment was conducted at room temperature for 60 minutes in the presence of DTPA.

Masses	17/1	8.6/1	2.8/1	0.58/1	modification
	%	%	%	%	
17428.98	-	-	23.56	38.55	unmodified
17443.98	-	-	2.25	4.60	+ 15
17457.02	-	-	-	2.07	+ 28
17473.95	-	-	14.33	25.08	+ 1 NO ₂
17489.03	-	-	2.62	2.12	+ 1 NO ₂ + 15
17518.97	-	7.54	12.59	10.15	+ 2 NO ₂
17534.02	-	2.18	2.90	-	+ 2 NO ₂ + 15
17547.97	-	3.55	2.07	-	+ 2 NO ₂ + 29
17563.95	-	19.17	11.21	3.10	+ 3 NO ₂
17579.00	-	5.46	2.16	-	+ 3 NO ₂ + 15
17592.96	-	4.95	-	-	+ 3 NO ₂ + 29
17608.95	15.86	22.00	7.95	-	+ 4 NO ₂
17624.95	2.30	4.88	-	-	+ 4 NO ₂ + 16
17637.95	3.59	2.58	-	-	+ 4 NO ₂ + 29
17653.94	21.06	12.19	3.71	-	+ 5 NO ₂
17669.94	5.24	2.12	-	-	+ 5 NO ₂ + 16
17682.90	2.55	-	-	-	+ 5 NO ₂ + 29
17698.89	11.28	3.22	-	-	+ 6 NO ₂
17714.91	3.26	-	-	-	+ 6 NO ₂ + 16
17743.87	9.06	-	-	-	+ 7 NO ₂
17759.89	5.78	-	-	-	+ 7 NO ₂ + 16

Table S7. Intact protein analysis by HPLC-HR-MS of ONOO⁻-nitrated Bet v 1 at 4 °C and at room temperature (~ 22 °C) for a reaction time of 100 min. The relative abundance of the identified protein molecular weights is demonstrated. The errors indicate the standard deviation of two experimental replicates.

ONOO ⁻ /Y (mol/mol)	Temp. (°C)	Relative MS abundance				
		Unmodified (17429 Da)	Oxidized (+ 16 Da)	Nitrated (+ n 45 Da)	Degraded (< 17000 Da)	Other (>17500 Da)
1	4	0.44 ± 0.07	0	0.43 ± 0.05	0	0.12 ± 0.04
	22	0.34 ± 0.07	0	0.47 ± 0.04	0	0.21 ± 0.04
5	4	0.12 ± 0.04	0	0.49 ± 0.09	0	0.36 ± 0.09
	22	0.09 ± 0.06	0	0.47 ± 0.06	0	0.43 ± 0.02
15	4	0	0	0.23 ± 0.12	0.34 ± 0.03	0.45 ± 0.09
	22	0	0	0.13 ± 0.14	0.44 ± 0.11	0.42 ± 0.01
30	4	0	0	0.05 ± 0.07	0.39 ± 0.18	0.63 ± 0.17
	22	0	0	0	0.86 ± 0.20	0.14 ± 0.20

Table S8. Reaction of Bet v 1 on filters and in aqueous solution with O₃/NO₂ at different reaction times. For the reaction in aqueous solution the NO₂ and O₃ concentrations were 100 ppb and for the reaction of Bet v 1 on filters the O₃ and NO₂ concentrations were 230 ppb. The values are the arithmetic mean ± SEM.

Tyr position	liquid (2h) ND _Y (%)	liquid (18 h)* ND _Y (%)	solid (6.5 h) ND _Y (%)	solid (15 h) ND _Y (%)	solid (24 h) ND _Y (%)	solid (48 h) ND _Y (%)
Y5	-	-	-	-	-	-
Y66	-	-	-	-	-	-
Y81 _(NO2)	8 ± 9 [#]	2 ± 1 [#]	0	0	0	2 ± 3
Y83 _(NO2)	18 ± 22 [#]	22 ± 7 [#]	32 ± 04	50 ± 29	66 ± 16	47 ± 12
Y120	-	-	-	-	-	-
Y150 _(NO2)	36 ± 38	5 ± 1	2 ± 2	5 ± 5	0	0
Y158 _(NO2)	6 ± 4	20 ± 1	3 ± 4	3 ± 1	25 ± 11	49 ± 12
Y158 _(OH)	5 ± 7	2 ± 3	46 ± 17	0	1 ± 2	5 ± 6
Y150 _(OH)	14 ± 10	6 ± 8	0	0	2 ± 1	0

* Condensed and partly dried protein due to long exposure time

[#] Y81 and Y81 were not clearly distinguished.

Table S9. Triplicate LC-MS/MS analysis of Bet v 1 on filters exposed to 230 ppb NO₂ and 230 ppb O₃ at a relative humidity of 92%. The nitration and oxidation sites of four different exposure times were analyzed with four parallel exposed filters for each time. (+++) means that the modified site is found in three of three analytical replicates with high intensities, (++) means that the modified site is found in two of three analytical replicates with high intensities or found in all three analytical replicates with low intensities, (+) indicates that the modified site is found in one of three analytical replicates with high or low intensities or found in two of three analytical replicates with low intensities, and (-) means that the modified site is not found in any of the runs. The mean intensity for the LC-MS/MS runs was $(5.04 \pm 0.92)10^6$ (arithmetic mean $\pm$ standard deviation). High intensity is defined as higher than 10% of the mean intensity ($\sim 10^5$ to 10^6) and low intensity is defined as lower than 1% of the mean intensity ($\sim 10^4$).

Exposure Time (h)	sample	Y66 +NO ₂	Y66 +OH	Y81 +NO ₂	Y81 +OH	Y83 +NO ₂	Y83 +OH	M139 + O	Y150 +NO ₂	Y150 +OH	Y158 +NO ₂	Y158 +OH
48	Bet_1	-	-	+	-	+++	-	+	-	-	+++	+++
	Bet_2	-	-	-	-	+++	-	-	-	-	+++	++
	Bet_3	-	-	-	-	+	-	+	-	-	++	+
	Bet_4	-	-	-	-	++	-	+	-	-	+++	-
mock	Bet_5	-	-	-	-	-	-	-	-	-	-	-
24	Bet_6	-	+	-	-	++	-	+++	++	-	+	-
	Bet_7	-	-	-	-	+++	-	-	-	-	+++	++
	Bet_8	-	-	-	-	++	-	-	-	-	+	+
	Bet_9	-	-	-	-	+++	-	+	-	+	+++	+++
mock	Bet_10	-	-	-	-	-	-	-	-	-	-	-
15.5	Bet_11	-	-	-	-	+++	-	+	-	-	+++	-
	Bet_12	-	+	-	-	+++	-	++	+	-	+	-
	Bet_13	-	-	-	-	+	+	+	-	-	-	-
	Bet_14	-	++	-	-	+++	-	+++	-	-	+++	-
mock	Bet_15	-	-	-	-	-	-	-	-	-	-	-
6	Bet_16	-	+	-	-	+++	-	++	-	-	-	++
	Bet_17	-	+	-	-	+	-	+	+	+	-	+
	Bet_18	-	+		-		-	+++		-		+++
	Bet_19	-	-	-	-	+	-	++	-	-	-	+++
mock	Bet_20	-	-	-	-	-	-	-	-	+	-	-
$\Sigma (+)$		0	7	0	0	34	1	22		3	26	21

Table S10. Triplicate LC-MS/MS analysis of Bet v 1 in aqueous solution exposed to 100 ppb NO₂ and 100 ppb O₃. The nitration and oxidation sites of two different exposure times were analyzed with two parallel experiments. (+++) means that the modified site is found in three of three analytical replicates with high intensities, (++) means that the modified site is found in two of three analytical replicates with high intensities or found in all three analytical replicates with low intensities, (+) indicates that the modified site is found in one of three analytical replicates with high or low intensities or found in two of three analytical replicates with low intensities, and (-) means that the modified site is not found in any of the runs. The mean intensity for the LC-MS/MS runs was $(5.04 \pm 0.92)10^6$ (arithmetic mean $\pm$ standard deviation). High intensity is defined as higher than 10% of the mean intensity ($\sim 10^5$ to 10^6) and low intensity is defined as lower than 1% of the mean intensity ($\sim 10^4$).

Exposure Time (h)	sample	Y66 +NO2	Y66 +OH	Y81 +NO2	Y81 +OH	Y83 +NO2	Y83 +OH	M139 + O	Y150 +NO2	Y150 +OH	Y158 +NO2	Y158 +OH
2	Bet 1	-	-	++	-	++	+	++	+++	+	+	+
	Bet 2	-	-	++	-	+++	-	++	+	+	+	-
mock	Bet m	-	-	-	-	-	-	-	-	-	-	-
18	Bet 3	-	-	+	-	++	-	-	++	-	+++	-
	Bet 4	-	-	++	-	+++	+	-	+	++	+	+
mock	Bet m	-	-	-	-	-	-	-	-	-	-	-
Σ (+)		0	0	7	0	10	2	4	7	4	6	2

Table S11. Intact protein analysis by HPLC-HR-MS of the O₃/NO₂-nitrated Bet v 1 at 100 ppb NO₂ and 100 ppb O₃ at ~47% RH. The errors represent the standard deviation of four experimental replicates. The sample indicated with 0 hour exposure is the mock-nitrated samples, which was not exposed to the gases.

Exposure time (h)	Relative MS abundance				
	Unmodified (17429 Da)	Oxidized (+ 16 Da)	Nitrated (+ n 45 Da)	Degraded (< 17000 Da)	Other (>17500 Da)
0	0.67 ± 0.01	0.24 ± 0.02	0	0	0.05 ± 0.02
3	0.48 ± 0.01	0.49 ± 0.01	0	0	0.03 ± 0.01
6.5	0.47 ± 0.02	0.51 ± 0.03	0	0	0.02 ± 0.02
15	0.45 ± 0.03	0.50 ± 0.02	0	0	0.05 ± 0.02
72	0.16 ± 0.06	0.69 ± 0.14	0	0	0.15 ± 0.09

Table S12. Intact protein analysis by HPLC-HR-MS of the O₃/NO₂-nitrated Bet v 1 with 80 ppb NO₂ and 20 ppb O₃. The relative humidity was as high that condensation on the filter took place (>98% RH). The errors represent the standard deviation of four experimental replicates. The sample indicated with 0 hour exposure is the mock-nitrated samples, which was not exposed to the gases.

Exposure time (h)	Relative MS abundance				
	Unmodified (17429 Da)	Oxidized (+ 16 Da)	Nitrated (+ n 45 Da)	Degraded (< 17000 Da)	Other (> 17500 Da)
0	0.78 ± 0.02	0.09 ± 0.02	0	0.05 ± 0.01	0.08 ± 0.01
1	0.77 ± 0.01	0.09 ± 0.02	0	0.07 ± 0.01	0.08 ± 0.01
19	0.47 ± 0.09	0.12 ± 0.03	0.28 ± 0.07	0.13 ± 0.19	0

SUPPLEMENTARY INFORMATION

S1 Protein digestion and HPLC-MS/MS analysis

0.1 mg of the nitrated Bet v 1 was dissolved in 0.1 mL water. As Bet v 1 does not contain cysteine residues, no dithiothreitol or iodoacetamide was added. 5 μ L 100 mM NH_4HCO_3 (Sigma-Aldrich, Steinheim, Germany), and 5 μ L trifluoroethanol (TFE, Sigma Aldrich, Steinheim, Germany) stock solution were added, and the protein was denatured for 1 h at 80 °C in a thermomixer (Thermomixer Comfort, Eppendorf, Germany). 60 μ L water and 20 μ L 100 mM NH_4HCO_3 were added to dilute and neutralize the solution (pH 7.5–8) before 2 μ L trypsin (Trypsin proteomics sequencing grade, Sigma-Aldrich, Taufkirchen, Germany) stock solution (1 μ g/ μ L in 50 mM acetic acid) were added to digest the protein, which occurred by incubation at 37 °C overnight (enzyme-to-substrate mass ratio 1:50). The samples were desalted using C_{18} spin tubes (Pep Clean C_{18} Spin Columns, Pierce Fisher Scientific, Schwerte, Germany) before HPLC-MS/MS analysis. C_{18} spin tubes were used according to the manufacturer's instructions, as already described elsewhere¹.

An Agilent HPLC-chip-MS/MS system (Agilent, Waldbronn, Germany) consisting of a nano pump (G2226A) with 4-channel micro-vacuum degasser (G1379B), a microfluidic chip cube with electrospray ionization (ESI) source (G4240-64000) interfaced to a Q-TOF mass spectrometer (6520; nominal mass resolution 20000 at a scan rate of 5 s^{-1}), a capillary pump (G1376A) with degasser (G1379B), and an autosampler with thermostat (G1377A), was used for analysis. All modules were controlled by Mass Hunter software (Rev. B.02.00, Agilent). A microfluidic reversed phase HPLC chip (Zorbax 300SB- C_{18} , 5 μm particle size, 75 μm inner diameter, and 150 mm length, Agilent) was used for peptide separation. Each chromatographic run was repeated three times with an injection volume of 0.2 μL . Eluents used were: 3% (v/v)

ACN (Chromasolve, Sigma, Seelze, Germany) in water/formic acid (0.1% v/v, Chromasolve, Sigma, Seelze, Germany) (Eluent A), and 3% water/formic acid (0.1% v/v) in ACN (Eluent B). Gradient elution was performed at a flow rate of 300 nL/min starting from 3% B, increasing to 40% B within 36 min, further increasing to 70% B at 38 min, and returning to initial conditions within 0.1 min, followed by column re-equilibration for 11.9 min before the next run. The ESI-Q-TOF instrument was operated in the positive ionization mode (ESI+) with an ionization voltage of 1900 V. Fragmentation of protonated ions was conducted using the auto MS/MS mode. Spectra were recorded at a rate of 3/s for MS and MS/MS mode. Database searches were performed using the Spectrum Mill software (Rev. A.03.03.084 and Rev. A.04.00.127, Agilent) against the database Swiss-Prot (www.expasy.ch). Protein identification and the search for PTMs including variable modifications such as nitrated tyrosine, oxidized methionine, hydroxylated tyrosine, and hydroxylated phenylalanine were performed with the Spectrum Mill software. The search criteria were as follows: database: SwissProtnew.fasta; digest: trypsin; species: all; maximum # missed cleavages: 2; precursor-ion mass tolerance: ± 20 ppm; fragment-ion mass tolerance: ± 50 ppm; masses: monoisotopic; minimum detected peaks: 4; minimum matched peak intensity: 50%, and minimum S/N: 25. Filtering criteria were: Protein score ≥ 13 ; peptide score > 10 and % SPI > 60 .

S2 HPLC-HR-MS analysis of intact Bet v 1.0101

Monolithic 150 x 0.20 mm PS-DVB capillary columns were prepared according to a previous published protocol.² Separations were performed with a capillary - HPLC system (Model UltiMate3000, Dionex Benelux) including a detector equipped with a 3 nL z-shaped capillary detection cell. Separations were generally accomplished at 55°C with gradients of acetonitrile (ACN) in 0.050% aqueous trifluoroacetic acid (TFA) at flow rates of 1 μ L/min. MS analysis was

performed with an Orbitrap mass spectrometer (Model Exactive, ThermoFisher Scientific), essentially under optimized conditions as published previously.³ A Nano Electrospray Source was utilized with a 20 μm id fused silica capillary and a tip drawn to 10 μm id (New Objective, Woburn, USA). The instrument was operated in positive ESI mode with a spray voltage of 1.5 kV, a capillary voltage of 95.0 V, a capillary temperature of 240 °C, a tube lens voltage of 180.0 V, and an Orbitrap target value of 10^6 . A maximum injection time of 500 ms was set and the MS parameters were optimized in the range of m/z 500–3000 by infusing a solution of myoglobin with a concentration of 1.3 pmol/ μl at resolutions of 7500–100 000 at m/z 400. Mass calibration was accomplished with the commercially available positive calibration solution for LTQ XL and LTQ Hybrids (Sigma Aldrich). The mass spectra were analyzed by 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.

S3 Experimental details for the nitration of Bet v 1 using O_3/NO_2 mixtures

A total of 17 experiments were conducted (see Table S2 for experimental conditions). For experiments 1-15, syringe filters (cellulose acetate membrane, 1.2 μm pore size, 30 mm diameter, sterile; FP30, Whatman GmbH, Germany) were pre-washed with ultrapure water and freeze-dried. The pre-treated filters were then loaded with 100 μL of Bet v 1 dissolved in 10 mM Na_2HPO_4 buffer at different concentrations to yield the loads listed in Table S1 and freeze-dried again. For each experiment five loaded syringe filters were prepared, of which four were used for the exposure experiment and the fifth filter was treated as a mock sample. For experiments 1-10 (setup 1, Fig. S1), the protein-loaded filters were exposed to mixtures of O_3 in synthetic air plus NO_2 and water vapor in nitrogen. Synthetic air, nitrogen (99.9995%), and ~ 5 ppmV NO_2 in N_2

were obtained from AIR LIQUIDE (Germany). NO₂ concentrations were adjusted by varying the source flow rate (5-20 mL min⁻¹). Ozone was produced from synthetic air passing by a partly shielded mercury lamp (Jelight Company, Inc., Irvine, US) at 1.5 L min⁻¹. Water vapor was generated by bubbling N₂ through a washing bottle filled with autoclaved high purity water at different temperatures and flow rates of 1.0 L min⁻¹ or 1.5 L min⁻¹ to adjust the relative humidity (RH). Experiments 1-6 were conducted at a lower relative humidity (RH = (47 ± 3)% (arithmetic mean ± standard deviation)), while experiments 7-10 were carried out at a high relative humidity (RH > 98%). In the latter experiments (experiments 7-10), condensation was observed in the tubing and on the filters.

For a better control of the RH at high values, an alternative experimental setup was used (experiments 11-15, 92% < RH < 98%; setup 2, Fig. S1). Here, the mixed O₃/NO₂ gas-flow was split; one part was passed through a Nafion® gas humidifier (MH-110-12F-4, PermaPure, Toms River, NJ, USA), while the other part remained dry. RH could be varied between 75% and 98% without condensation in the tubing by adjusting the ratio between the dry and humidified air flows. During the experiments, the standard deviation from the set RH values was < 1%. NO₂ and O₃ concentrations and RH were monitored using commercial instruments (NO_x analyzer, 42i-TL, Thermo Scientific, Germany; ozone analyzer, 49i, Thermo Scientific, Germany; RH sensor FHA 646-E1C with ALMEMO 2590-3 instrument, Ahlborn, Mess- und Regelungstechnik, Germany). The RH sensor has a working range of 5% - 98% RH with an accuracy of ± 2% RH at 25°C. After the respective exposure times, the proteins were extracted from the filters with 0.6 mL ultrapure water. Further analysis was performed using the extractable and soluble fraction. Protein recovery, determined by a BCA assay (see below), was (65.5 ± 17.6)% for exposed protein, and (91.5 ± 5.8)% (arithmetic mean ± standard deviation) for non-exposed protein.

To further investigate the role of the phase-state of the protein for the nitration kinetics, experiments 16-17 were conducted using setup 3 (Fig. S1). Here, O₃/NO₂ gas mixtures were bubbled directly through Bet v 1 solution (for concentrations see Table S2) at flow rates of 2 mL min⁻¹ to ensure saturation of the bulk solution with NO₂ and O₃. Recovery depended strongly on reaction time and was determined to be (78 ± 1)% for 2 h of exposure, and (20 ± 11)% for 17 h of exposure.

S4 Kinetic model simulations

For interpretation of the obtained experimental results for the reaction of dissolved protein in aqueous solution, the second-order reaction rate coefficients (k_{BR}) with the below rate equations [1a-c] were considered:

$$\frac{d[\text{Tyr}]}{dt} = -k_{BR,a}[\text{O}_3][\text{Tyr}] \quad [1a]$$

$$\frac{d[\text{ROI}]}{dt} = k_{BR,a}[\text{O}_3][\text{Tyr}] - k_{BR,b}[\text{NO}_2][\text{ROI}] \quad [1b]$$

$$\frac{d[\text{Tyr-NO}_2]}{dt} = k_{BR,b}[\text{NO}_2][\text{ROI}] \quad [1c]$$

The bulk concentrations of O₃ and NO₂ in an aqueous solution can be estimated by using the Henry's law constant of $K_{\text{sol,cc,O}_3} \approx K_{\text{sol,cc,NO}_2} \approx 10^{-5} \text{ mol cm}^{-3} \text{ atm}^{-1}$.⁴ Here, we assumed a maximal ND of 0.28, which is equivalent to 2 fully nitrated Tyr residues in Bet v 1. The above differential equations were numerically solved using a Matlab software with varying $k_{BR,a}$ and $k_{BR,b}$ to fit to the experimental data. The formation of a ROI, most likely a Tyr radical, was found to be the rate-limiting step, which is consistent with previous studies^{5,6}, and $k_{BR,a}$ could be well-constrained to be $4 \times 10^{-17} \text{ cm}^3 \text{ s}^{-1}$ ($= 2.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$). This value is within the range of reported value for bulk reaction between amino acid and ozone in water, which vary by three orders of magnitude in

$10^4 - 10^7 \text{ M}^{-1} \text{ s}^{-1}$.^{4, 7-11} Rate constant $k_{\text{BR},b}$ was found to be $>10^{-15} \text{ cm}^3 \text{ s}^{-1}$, but could not be well-constrained as the modeled ND is not sensitive to $k_{\text{BR},b}$ if $k_{\text{BR},b}$ is $>10^{-15} \text{ cm}^3 \text{ s}^{-1}$.

To analyze and interpret the obtained experimental data for the heterogeneous reaction of Bet v 1 on filters at $<92\%$ RH, the kinetic multi-layer model for aerosol surface and bulk chemistry (KM-SUB¹²) was applied. KM-SUB explicitly resolves gas-particle interactions between O_3 , NO_2 , and protein including reversible adsorption, surface-bulk exchange, bulk diffusion, and surface and bulk reactions. The required kinetic parameters include Henry's law constants and second-order bulk reaction rates were assumed to be same as for the aqueous-phase reaction case. The other parameters are surface accommodation coefficients and desorption lifetime, which were assumed to be same as those determined in the previous study of nitration of bovine serum albumin.⁵ The total surface area of deposited Bet v 1 was assumed to be the same as the geometric surface area of the filter for simplicity.

At an $\text{RH} < 92\%$ the physical state of protein is (semi-)solid with typical bulk diffusion coefficient of oxidants (D_b) on the order of $10^{-10} - 10^{-8} \text{ cm}^2 \text{ s}^{-1}$.⁷ In Figure 4a the gray shaded area shows the range of simulated ND assuming $D_b = 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ and gas-phase O_3 and NO_2 concentrations of 100 – 470 ppb. The model captured the observed data fairly well (Fig 4a). When an increased D_b value of $10^{-5} \text{ cm}^2 \text{ s}^{-1}$ was used that is a characteristic magnitude of D_b in liquid, it explains the observed enhanced ND for protein on filter under condensing conditions at $\text{RH} > 98\%$ (Fig. 4a).

S5 Detailed discussion of the results for heterogeneous and homogeneous nitration of Bet v 1 using O_3/NO_2 mixtures

1) Reaction of gaseous O_3/NO_2 with Bet v 1 on filters at 45-92% RH

The results of the corresponding experiments are illustrated in the grey shaded area of Figure 4a. The ND ranged from 2% to 4% for Bet v 1 on filters (experiments 1-6 and 11-14, Table S2). Variation of RH from 47% to 92% did not significantly influence the ND.

Intact protein analysis by HPLC-HR-MS showed mainly oxidation ($\Delta m = 16$ Da) for the heterogeneous nitration at O_3 and NO_2 mixing ratios of 100 ppb and low RH (~45%) (experiments 1-6) (see Table S11). After 70 h of exposure the relative abundance of oxidized protein reached more than 60%.

For low RH (~45%) experiments we did not observe any nitrated tyrosine containing peptides in the HPLC-MS/MS analysis. In Figure 4b the HPLC-MS/MS analyses of tryptic peptides of Bet v 1 exposed to O_3/NO_2 at a relative humidity of 92% (experiments 11-15, Table S2) showed that four or five of seven tyrosine residues could be identified with an amino acid sequence coverage of $(71.1 \pm 3.4)\%$ (arithmetic mean $\pm$ standard deviation) (for details see Table S8). Y83 and Y158 were found in nitrated form in all samples, while Y158 was also present in oxidized form (+ OH) in some samples. Y150 was found nitrated (+ NO_2) or oxidized (+ OH) in some samples, Y66 was found in oxidized form (+ OH), and methionine at position 139 was also found in oxidized (+ O) form (for details see supplementary Table S9). However, Y83 and Y158 were the preferred sites for the heterogeneous nitration of Bet v 1 for different reaction times (Fig. 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) supporting the assumption of reactive oxygen intermediates to be involved in the first step of the nitration reaction.⁵

2) Reaction of gaseous O_3/NO_2 with Bet v 1 on filters at >98% RH

The ND reached up to 6% after 19 hours for >98% RH (water condensation on filters, experiment 7-10, Table S2).

Intact protein analysis by HPLC-HR-MS of filter samples with high RH under water condensing conditions (>98% RH, experiments 7 + 9), the addition of one or two nitro groups was observed, along with a smaller amount of oxidation (Table S12). Thus, oxidation seems to be favored under low RH conditions, whereas under high RH conditions nitration appears to be more prominent.

3) Reaction of dissolved O_3/NO_2 with dissolved Bet v 1 in aqueous solution

The ND for the homogeneous reaction in aqueous solution (both the gases and the protein are dissolved in an aqueous solution, experiments 16-17, Table S2) reached up to 22% after 17 hours.

Exemplary HPLC-MS/MS analysis of O_3/NO_2 -nitration in aqueous solution (experiments 16-17) generally resulted in a similar nitration pattern as for the heterogeneous reaction, i.e., Y158 and Y83 or Y81 were the preferred sites (Table 2), suggesting the similar reaction pathways were followed in both heterogeneous and homogeneous nitration by O_3/NO_2 . One sample of the aqueous solution experiments (2 h of exposure, see Table S8) differed from the other samples. Here, Y150 was observed to be the most efficiently nitrated residue and the pattern was more in line with the pattern of $ONOO^-$ nitration. This discrepancy, which may suggest different rates for competing reaction pathways, will be investigated in future studies.

Y158 is located at the C-terminus of the Bet v 1 molecule within a coil structure motif. As coil structures 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.¹³ Y83 and Y81 are located at the hydrophobic cavity and might be a preferred site, due to 1) the stabilization of Tyr $\cdot$ in hydrophobic environments, and 2) because most of the reactive nitrogen compounds are hydrophobic gases.¹⁴

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