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Nitration of pollen aeroallergens by nitrate ion in conditions simulating the liquid water phase of atmospheric particles

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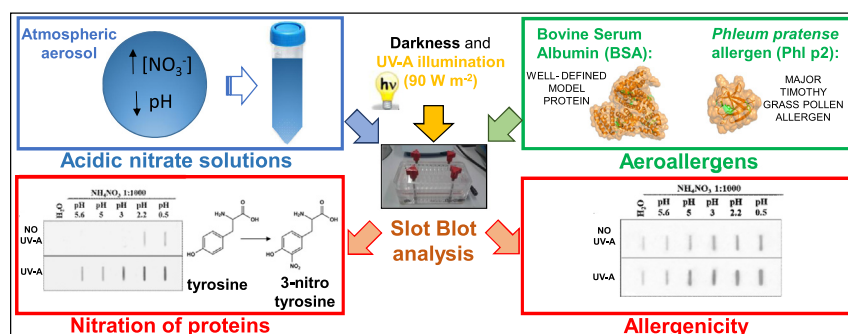
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HIGHLIGHTS

- Atmospheric reactivity may affect pollen protein allergenicity
- We studied protein nitration in nitrate solutions simulating the liquid phase of PM
- Nitrate ion, a major component of PM, can induce protein tyrosine nitration
- Results show that acidic pH and UV-A irradiation enhance the nitration reaction
- Protein nitration increased the allergenicity despite protein degradation

GRAPHICAL ABSTRACT



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ABSTRACT

Pollen aeroallergens are present in atmospheric particulate matter (PM) where they can be found in coarse biological particles such as pollen grains (aerodynamic diameter $d_{ae} > 10 \mu\text{m}$), as well as fragments in the finest respirable particles ($\text{PM}_{2.5}$; $d_{ae} < 2.5 \mu\text{m}$). Nitration of tyrosine residues in pollen allergenic proteins can occur in polluted air, and inhalation and deposition of these nitrated proteins in the human respiratory tract may lead to adverse health effects by enhancing the allergic response in population. Previous studies investigated protein nitration by atmospheric gaseous pollutants such as nitrogen dioxide and ozone. In this work we report, for the first time, a study on protein nitration by nitrate ion in aqueous solution, at nitrate concentrations and pH conditions simulating those occurring in the atmospheric aerosol liquid water phase. Experiments have been carried out on the Bovine serum albumin (BSA) protein and the recombinant *Phleum pratense* allergen (Phl p 2) both in the dark and under UV-A irradiation (range $4\text{--}90 \text{ W m}^{-2}$) to take into account thermal and/or photochemical nitration processes. For the latter protein, modifications in the allergic response after treatment with nitrate solutions have been evaluated by immunoblot analyses using sera from grass-allergic patients. Experimental results in bulk solutions showed that protein nitration in the dark occurs only in dilute nitrate solutions and under very acidic conditions ($\text{pH} < 3$ for BSA; $\text{pH} < 2.2$ for Phl p 2), while nitration is always observed (at $\text{pH} 0.5\text{--}5$) under UV-A irradiation, both in dilute and concentrated nitrate solutions, being significantly enhanced at the lowest pH values. In some cases, protein nitration resulted in an increase of the allergic response.

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1. Introduction

Allergenic proteins from pollen are widely diffused in air during the flowering periods of plants (Buters et al., 2008 and 2010), and their content in the atmosphere varies according to climate, geography and vegetation of the different areas (Miguel et al., 2006; D'Amato et al., 2007; Blando et al., 2012).

The primary carrier in air of pollen aeroallergens are the pollen grains, which have a typical aerodynamic size of 15–40 μm when intact. However, strong evidences support the existence of pollen allergenic proteins in the atmosphere as individual molecules separated from pollen and associated to atmospheric particles (PM) of sizes much smaller than intact pollen grains (De Linares et al., 2007 and 2010; Fernández-González et al., 2010). Indeed, pollen allergenic proteins have been found in PM₁₀, PM_{2.5} (also known as “PM respirable fraction”) as well as in the finest submicrometric particle fraction (PM₁ and finer particles) (Taylor et al., 2004; Bacci et al., 2006; D'Amato et al., 2007).

Pollen grains at maturity contain a large number of different proteins, and it is well known that some pollen proteins can act as allergens and belong principally to the protein families of profilins, expansins, calcium-binding proteins, thaumatin-like proteins and non-specific lipid transfer proteins (Breiteneder and Ebner, 2000; Radauer and Breiteneder, 2006). The presence of pollen allergenic proteins in atmospheric fine PM can have several consequences of concern for the diffusion and growth of the allergic diseases (D'Amato et al., 2007; Sofiev and Bergmann, 2013), due to: (i) a longer atmospheric residence time of fine particles and a long range transport of pollen allergenic proteins; (ii) clinical effects in the deeper respiratory airways due to the penetration capacity of the fine particles (e.g. PM_{2.5}); (iii) post-translational modifications that can occur in pollen allergenic proteins contained in the PM. The latest point, which refers to possible chemical transformations of pollen allergenic proteins in atmospheric PM, is thought to be very important to explain the observed increase of allergic pathologies in urban environments associated to high concentrations of atmospheric pollutants and PM (Farkhondeh and Ahmad, 2011).

Among the various chemical modifications that allergenic proteins may undergo in the atmosphere, the nitration of the aromatic ring of the amino-acid tyrosine (yielding to 3-nitrotyrosine) has been proposed to be very relevant in allergic pathologies due to the potential enhancement of the allergenicity of such nitrated proteins (Walcher et al., 2003; Gruijthuisen et al., 2006; Zhang et al., 2011; Shiraiwa et al., 2012a and 2012b). Previous studies have investigated the nitration of proteins in air by gaseous pollutants, such as nitrogen dioxide ($\cdot\text{NO}_2$) and ozone (O_3) (Shiraiwa et al., 2012b; Reinmuth-Selzle et al., 2014). Franze et al. (2005) showed that major allergen from birch Bet v 1 is effectively nitrated in the presence of $\cdot\text{NO}_2$ and O_3 , and that such nitration affects the allergenicity of the birch pollen. It has been shown that activated aromatic compounds such as phenol and cresols can be nitrated (yielding to the corresponding nitrophenols and nitrocresols) in the liquid water phase of atmospheric PM by nitrate ions (NO_3^-) in thermal and/or photochemical processes (Vione et al., 2006; Lüttke et al., 1997). Tyrosine residues in proteins may feature the same kind of reactivity, therefore providing alternative pathways for protein nitration in atmospheric PM. However, protein nitration in the bulk PM phase, due to the reaction of proteins with nitrate ions contained in PM, has never been investigated so far.

The nitrate ion (NO_3^-) is one of the most abundant components of PM. It corresponds to 8–16% of PM_{2.5} total mass as annual average at urban, rural and kerbside sites in Europe, with the highest values (25–30% of PM_{2.5} mass) in winter, when it forms in the atmospheric condensed phase from oxidation of NO_x gas pollutant emitted by combustion sources (Putaud et al., 2010). Total inorganic ions, mainly including sulphate and ammonium in addition to nitrate, can explain about 50% of total PM_{2.5} mass (Putaud et al., 2010).

PM can be present in the atmosphere in solid, liquid and a mix of solid–liquid states according to the hygroscopic characteristics of atmospheric particles (Casati et al., 2015; D'Angelo et al., 2016) and environmental relative humidity (RH). Adsorption of water molecules by solid PM results in particle deliquescence at a given relative humidity (deliquescence relative humidity; DRH), and in these conditions the water phase can feature very high ions concentrations, close to saturation of soluble salts such as ammonium nitrate (NH_4NO_3) and ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$). The acidity of this PM liquid water phase is strongly dependent on the presence of not-neutralized acid components (mainly NO_3^- and SO_4^{2-}), and on the PM liquid water content (the mass of liquid water adsorbed by unit particle mass μg^{-1}). The liquid water phase of atmospheric PM can be highly acidic, and very low pH values (0–2) have been reported in literature for PM of different urban and suburban sites worldwide (Behera et al., 2013; Squizzato et al., 2013). Therefore, the liquid water phase of atmospheric PM close to the deliquescence point can feature ‘extreme’ conditions, in terms of ions concentrations and pH, that are expected to be crucial for chemical and photochemical reactions occurring in such liquid phase.

Prompted by the above considerations, we have carried out a batch study to investigate if protein nitration can occur in the presence of nitrate, under the characteristic pH and concentration conditions that really occur in the liquid water phase of atmospheric particles.

The experiments have been carried out using two kinds of allergenic proteins: the Bovine serum albumin (BSA) protein and the *Phleum pratense* allergen Phl p 2. BSA is a well-defined model protein commonly used in laboratory experiments. Even though BSA is not a pollen allergen, it has been detected in the atmosphere, and it can therefore be considered an aeroallergen (Choi et al., 2009; Voltolini et al., 2013). Phl p 2 is a major timothy grass (*Phleum pratense*) pollen allergen. *Phleum pratense* is an important species of the *Poaceae* family due to its geographical diffusion and its clinical significance for pollen allergy. The degree of protein nitration in acidic nitrate solutions has been determined by a biochemical test (Slot Blot analysis). Protein nitration has been investigated in the dark and under UV-A irradiation, to take into account both thermal and photochemical reaction pathways that can occur in the atmosphere. In order to evaluate changes in the allergenicity of Phl p 2 after treatment with the nitrate solutions in the different conditions, immunoblotting analysis were conducted using a sera mix from grass pollen allergic patients.

It is important to note that the aim of this preliminary work was not to quantify the degree of protein nitration nor to investigate on the molecular nitration mechanism, but it was to qualitatively evaluate if nitration of proteins can occur solely in the presence of the nitrate ion as nitrating agent in acidic conditions such as those typically occurring in the liquid water phase of atmospheric PM. In this respect, we believe that our experimental results may be important as they add new knowledge on the relevance of protein nitration occurring in the bulk PM liquid phase. Likewise, we are interested in evaluating the allergenic response of the treated proteins, also considering that in the acidic conditions featured by the experiments proteins may undergo further modifications such as degradation or aggregation providing other routes to alter the allergenic response.

2. Material and methods

2.1. Simulation of ionic concentrations and acidity in the liquid water phase of atmospheric PM

The concentrations of soluble ions in the atmospheric aerosol liquid water phase ($\text{mol kg}^{-1} \text{H}_2\text{O}$ and $\text{mol l}^{-1} \text{sol}$) and its acidity (pH) were simulated by using the E-AIM (Extended Aerosol Inorganic Model) model (<http://www.aim.env.uea.ac.uk/aim/aim.php>) (Wexler and Clegg, 2002), which calculates the thermodynamic properties of highly concentrated aqueous solutions of inorganic salts relevant for the

atmosphere. In particular, the current online version of E-AIM, Model II, has been used for calculations (Clegg et al., 1998).

As input data we referred to the average chemical composition of PM_{2.5} in the summer season (June–Sept: when the maximum of grass pollen allergenic proteins is expected in the atmosphere due to the flowering) for atmospheric concentrations ($\mu\text{g m}^{-3}$) of the most abundant water soluble inorganic ions (nitrate, sulphate and ammonium ions) measured in the PM_{2.5} of Milan, Northern Italy (Perrone et al., 2010 and 2012).

Ammonia (present as ammonium ion in the PM) is the major atmospheric neutralizing agent, but because of typically high amount of the acidic PM components sulphates and nitrates in polluted areas, ammonia may not be concentrated enough for complete neutralization, making PM acidic. The neutralization ratio (NR) (Bencs et al., 2008).

$$\text{NR} = [\text{NH}_4^+] / ([\text{SO}_4^{2-}] + [\text{NO}_3^-])$$

is used to describe the aerosol acidity, expressing the degree of neutralization of sulphate and nitrate by ammonium (expressed as equivalent).

According to studies of the ionic balance of PM_{2.5} in Milan (Perrone et al., 2010), as well as to studies reported in literature for other urban sites of North Italy (Squizzato et al., 2013), three different neutralization conditions were considered: complete ammonium neutralization (NR = 1.000); very low ammonium deficit (0.2% of ammonium deficit NR = 0.998); 10% of ammonium deficit (NR = 0.900).

The input data entered in the E-AIM model (ion concentrations, NR) and output results of the model (DRH at 25 °C; amount of liquid water, dissolved ions and pH at DRH) are all listed in Table S1. pH is used to describe PM acidity and represents the hydrogen ion activity in the aqueous solution.

$$\text{pH} = -\log(\gamma_{\text{H}} \times x_{\text{H}^+})$$

where γ_{H} is the hydrogen ion activity coefficient and x_{H^+} is the aqueous mole fraction of dissociated H^+ .

At DRH, the water content of particles is low (H_2O is 0.52–0.61 as mole fraction), and the ion concentrations in the aqueous solution is maximum. At DRH, the calculated nitrate concentration in the liquid water phase of atmospheric PM was 13.1–13.9 mol kg^{-1} H_2O , which is close to the saturation of ammonium nitrate (ammonium nitrate saturation is ~ 24 mol kg^{-1} H_2O at 20 °C) (Lide and Haynes, 2010).

Highly acidic conditions (pH = 2.48) of the liquid water phase of atmospheric PM were calculated at DRH even with a very low (0.2%; NR = 0.998) ammonium deficit. For a higher ammonium deficit (10%), the acidity values were still higher (pH = 0.44) (Table S1).

2.2. Aqueous solutions prepared for laboratory experiments of protein nitration in bulk solutions

Two types of aqueous solutions were prepared:

-Concentrated solution (close to the NH_4NO_3 saturation condition) (1:1): they were prepared by referring to the ionic concentrations and pH of the liquid water phase of atmospheric PM at DRH, as simulated by E-AIM model (see Section 2.1 and Table S1).

-Diluted solutions (1:100 and 1:1000): they were prepared to simulate a higher liquid water content of atmospheric particles and, correspondingly, diluted ionic concentrations in the aqueous solution. This would occur when RH > DRH, and water vapor goes on condensing on atmospheric particles that can serve as nuclei for cloud or fog droplets which are in the size range of coarse PM (typically ~ 10 μm diameter, but ranging greatly in size from 1 to 100 μm). In fact, a dilution 1:100 would be for a fine atmospheric particle (1 μm) growing 4.7 times its diameter (4.7 μm), thus $4.7^3 = 104$ times its initial volume, due to water uptake. A dilution 1:1000 would be for the same fine atmospheric particle (1 μm) growing 10 times its diameter (10 μm), thus $10^3 = 1000$ times its initial volume, due to further water uptake. Clearly, dilution

of the solution due to the water uptake may cause a significant change in pH. For example, for NR = 0.900 (10% of ammonium deficit) the pH is equal to 0.44 at the DRH, and pH increases to 2.39 and 3.39 for 1:100 and 1:1000 dilutions, respectively (this estimation neglects all other processes eventually occurring in the solution, such as phase partitioning and dissociation equilibria).

Solutions were prepared by dissolving in ultrapure water (Milli-Q®) the required amounts of ammonium nitrate (NH_4NO_3 ; $\geq 99.0\%$, Sigma Aldrich), and subsequently adjusting the acidity at various pH values (from 5.6 to 0.5) by adding sulphuric acid (H_2SO_4 ; 95–98%, VWR). The pH of the solutions was measured by a glass electrode (pH Meter HI 2211; Hanna Instruments, precision ± 0.01). All the solutions were stored in sterile plastic falcons (25 ml) in the dark at -18 °C until their use for protein nitration experiments.

2.3. Proteins: BSA and recombinant Phl p2

We used two different proteins: Bovine Serum Albumin (BSA) and recombinant Phl p2.

Commercial BSA standard protein solution supplied by Sigma Aldrich was dissolved in water at the concentration of $0.5 \mu\text{g } \mu\text{l}^{-1}$.

Phl p2 is one of the most relevant allergenic proteins present in timothy (*Phleum pratense*) belonging to group 2/3 of grass pollen allergens. For this study, a recombinant Phl p2 protein (gently provided by Dr. Gianni Mistrello and Dr. Stefania Zanotta, Laboratory of Research and Development, Lofarma S.p.A. Viale Cassala 40 - 20143 Milano, Italy) was used. It was obtained after production in *E. coli* and subsequent purification. IgE-binding capacity of the recombinant protein was tested by using sera from people allergic to timothy. Recombinant protein Phl p2 was diluted in water at $0.5 \mu\text{g } \mu\text{l}^{-1}$.

2.4. Dark and light irradiation conditions

Protein nitration in acidic nitrate solutions was investigated both in the dark (no irradiation) and under illumination with UV-A. $0.5 \mu\text{g}$ of BSA or Phl p2 proteins were transferred in single well of an optically clear UV transparent microtiter plate containing 20 μl of each of the nitrate solutions prepared before. Immediately microtiters were transferred under the UV-A and incubated for 10 min (minimum experimental time) and 30 min. UV irradiation at 365 nm was conducted using a transilluminator Vilber Lourmat TFX-35 modified in order to carry lamps Vilber Lourmat with same emission spectrum (ultraviolet light with wavelengths ranging from 315 to 400 nm, maximum emission at 365 nm) and chosen intensity. Different irradiance were used: 4, 16, 30 and 90 W m^{-2} . Not UV treated samples were placed in a box completely in the dark near the treated samples under the lamps.

All experiments with BSA and Phl p2 have been repeated at least three times (3 to 5) to confirm the results.

2.5. Patients and preparation of a sera mix

Sera from 13 adult patients mono-sensitized to grass pollen, collected by the Department of Allergology of the Clinica San Carlo (Paderno Dugnano, Milan, Italy), were utilized for this study. All patients had previously undergone routine diagnostic analyses and gave an informed written consent to the use of their stored sera for scientific purposes in an anonymous form. *Phleum*-specific IgE levels, measured by ImmunoCAP (Phadia, Uppsala, Sweden), ranged between 8.13 and 88.1 Ku l^{-1} . Sera from 3 healthy, nonatopic adults (showing negative clinical history and negative SPT with commercial airborne allergens extracts) were used as controls. Sera were pooled to carry out all immunochemical analyses and the mix was aliquoted and stored at -20 °C until use.

2.6. Slot Blot analysis to assess protein nitration and allergenicity

Slot Blot is a simple technique for immobilizing proteins on a membrane (nitrocellulose, polyvinylidene difluoride or PVDF, nylon). In our case a dissolved sample containing proteins was filtrated through a nitrocellulose membrane (0.22 μm ; Hybond-C membrane, Amersham) by applying vacuum after incubation with acidic nitrate solutions (in the dark and under UV-A irradiation as described in Section 2.4), to adsorb the treated proteins into the membrane.

Equal amounts of protein were loaded on each well (0.5 μg) and bound to nitrocellulose membranes. In order to check that the same amount of proteins was loaded in each well, membranes were first stained with Ponceau S Staining solution [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid]. Ponceau S solution is a rapid and reversible staining method used for the detection of proteins with a limit of detection of about 250 ng of transferred protein. Ponceau S is a negative stain, which binds to the positively charged amino groups of the protein. It also binds non-covalently to non-polar regions in the protein. Once treated, background stain can be easily removed by a quick rinse with water.

Membranes were then treated in two different ways. A set of them, in order to assess protein nitration, were incubated for 1 h with antibodies against 3 nitro-tyrosine produced in rabbit (Sigma Aldrich) diluted 1:5000 in TBS-T [20 mM Tris, 150 mM NaCl and 0.05% (v/v) Tween 20, pH 7.5] containing 1% (w/v) nonfat dry milk powder and, after washing, for 1 h with goat anti-rabbit IgG-Alkaline Phosphatase antibodies (Sigma Aldrich) diluted 1:15,000 in TBS-T 1% (w/v) nonfat dry milk powder. The signals were detected using Sigma FAST™ BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets. BCIP was used in conjunction with NBT for the colorimetric detection of alkaline phosphatase activity. This substrate system, when incubated with alkaline phosphatase, produces an insoluble NBT diformazan product that is easily observable for its purple colour. The final colour depends primarily on the abundance of target antibody.

The other set of membranes, in order to evaluate the allergenicity of proteins, were simultaneously treated overnight with pooled sera (diluted 1:10; PBS Tween 0.1) and then with polyclonal anti human IgE-secondary antibody conjugated with peroxidase (Sigma-Aldrich). The signals were detected using the SuperSignal West Dura detection kit (Pierce). SuperSignal West Dura Substrate is a highly sensitive enhanced chemiluminescent substrate for detecting horseradish peroxidase (HRP) on immunoblots. The extremely intense signal output enables detection in the high femtogram range of the antigen. The sensitivity, intensity and duration of the signal allow for easy detection of HRP using photographic or other imaging methods. Immunoreactive bands were visualized on an X-ray film (Kodak).

2.7. SDS PAGE and gel staining by silver stain

In order to assess the quality of the proteins and their degradation, 0.10 μg of each sample, treated as reported above, were loaded in 14% sodium dodecyl sulphate polyacrylamide gel and submitted to electrophoresis (SDS-PAGE). Then, gels were stained with Silver Stain kit (Bio-Rad, Italy) following manufacturer's protocol.

3. Results

3.1. Nitration of the BSA protein under UV-A irradiation and in the dark

BSA is a globular protein of 67 kDa commonly used as protein standard in laboratory experiments. It was also used in previous studies to investigate the chemical kinetics of the nitration of aerosolized proteins by atmospheric gas pollutants (Shiraiwa et al., 2012b). The protein contains 21 Tyr residues that may be nitrated in the reaction conditions.

Several experiments were performed to assess BSA nitration in acidic nitrate solutions by changing the nitrate concentration (1:1; 1:100;

1:1000; see Table 1 for the specification of nitrate concentrations), the pH value (pH ranging from 5.6 to 0.5) and the reaction time (ranging from 10 min, minimum experimental time, to 30 min), both in the dark and under UV-A irradiation (irradiance of 4, 16, 30 and 90 W m^{-2}). It is important to note that the maximum reaction time (30 min) considered in this study is sufficiently short compared to the typical atmospheric residence time of tropospheric aerosols, that is in the order of hours to days for fog/cloud droplets, and of days to weeks for fine particles (Seinfeld and Pandis, 2006). Therefore, such reaction time can be considered representative (as a lower bound limit) to look at chemical processes occurring in the aerosol liquid water phase. As for the irradiance of the UV-A lamps, we can note that the solar UV-A irradiance at middle latitude ($\sim 53^\circ$) in summer is about 45 W m^{-2} as average summer value at noon, with much higher values at lower latitude and in single days (Seckmeyer et al., 2008). Therefore, the maximum UV-A irradiance applied in the experiments carried out under irradiation (90 W m^{-2}) is representative of a sunny and clear summer day, with a peak in solar irradiance, whereas the minimum irradiance (4 W m^{-2}) is low compared to normal solar irradiance conditions.

The results of the BSA nitration experiments are summarized in Fig. 1 and Fig. 2, which report the Slot Blot analysis in the different experimental conditions discussed above. Hereafter we will refer (qualitatively) to the degree of protein nitration as related to the intensity of the bands in the Slot Blot analysis.

The degree of BSA nitration increased progressively by increasing the reaction time (see Fig. 1) thus evidencing the reaction rate over this time range. Since the signal was always most intense at the longest reaction time considered (30 min) all the reported results are referred to this reaction time.

In the experiments carried out under UV-A irradiation the effects of increasing the irradiance (4, 16, 30 and 90 W m^{-2}) on the BSA nitration are shown in Fig. 2. The results clearly show that the degree of protein nitration significantly increased by raising the lamp irradiance, with a noticeable effect also given by nitrate concentration and pH. Indeed, the degree of BSA nitration under irradiation always increased by shifting to more acidic pHs, and to higher nitrate concentrations. It is worth noting that protein nitration is also observed by irradiating the protein with the lamp of lowest irradiance (4 W m^{-2}), provided that the nitrate concentration is sufficiently high and the pH is sufficiently low (for example nitration is detected for the 1:100 nitrate solution and pH = 2.2, or for the 1:1 nitrate solution and pH = 3) (Fig. 2). As the irradiance raised, the signals corresponding to protein nitration were progressively detected for lower nitrate concentrations and higher pH values. In particular, BSA nitration was observed under the maximum UV-A irradiance applied (90 W m^{-2}) for the solutions at all concentration values (1:1000, 1:100 and 1:1) and over the pH range 5–0.5 (time: 30 min) (Fig. 1 and Fig. 2).

Nitration of BSA in the dark (see the left column of Fig. 1) was observed for the 1:1000 solution over the pH range 5–0.5 (reaction time: 30 min). The degree of protein nitration progressively increased at more acidic pH. Notably, the degree of BSA nitration in the dark significantly decreased by increasing the concentration of the nitrate solution. Indeed, a weak signal was still observed for the 1:100 solution at pH 0.5, whereas no BSA nitration was detected for the 1:1 solution at any pH investigated. The integrity of the BSA protein after treatment with the acidic nitrate solutions both in the dark and under UV-A irradiation

Table 1

Acidic nitrate solutions used in the experiments of protein nitration. Nitrate ionic concentrations (mol kg^{-1}) and pH values simulate different conditions occurring in the atmospheric aerosol liquid phase.

Solutions			pH 5.6	pH 5.0 ^a	pH 3.0 ^a	pH 2.2 ^a	pH 0.5 ^a
dil 1:1	NH_4NO_3	mol kg^{-1}	13.30	13.37	13.15	13.07	13.90
dil 1:100	NH_4NO_3	mol kg^{-1}	0.13	0.13	0.13	0.13	0.14
dil 1:1000	NH_4NO_3	mol kg^{-1}	0.013	0.013	0.013	0.013	0.014

^a Solutions were acidified with H_2SO_4 .

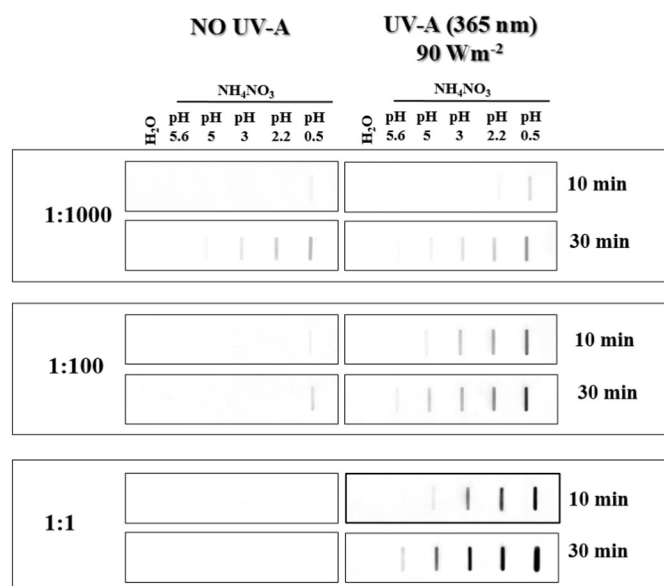


Fig. 1. BSA nitration evaluated using Slot Blot membranes probed with the antibody against 3-nitrotyrosine, after treatment of the protein with NH_4NO_3 solutions at different pH (5.6, 5.0, 3.0, 2.2, 0.5), and different nitrate concentrations (1:1, 1:100, 1:1000; see Table 1 for the specification of nitrate concentrations), in the dark (no UV-A), as well as under UV-A light (365 nm; 90 W m^{-2}) irradiation for 10 or 30 min. Slot Blot membranes are representative of 5 different experiments.

has been assessed by the SDS-PAGE analysis reported in the Fig. S1 of the Supplementary materials. At 30 min, BSA degradation was not observed for any nitrate concentration and pH when the reaction was carried out in the dark or with the smallest irradiance (4 W m^{-2}), whereas it was still low at the lowest pH (2.2–0.5) and the maximum irradiation (90 W m^{-2}).

In summary, nitration of BSA was observed both in the dark and under irradiation experiments. In both cases, the degree of protein nitration increased in the more acidic conditions. Moreover, under

irradiation BSA nitration largely increased as a function of the nitrate concentration, and a very large protein nitration degree was observed for the 1:1 nitrate solution (Fig. 1). On the contrary, in the dark, the largest degree of protein nitration was detected for the lowest nitrate concentration (1:1000), whereas nitration did not appear to occur for more concentrated solutions. This counterintuitive trend will be further discussed in Section 4.

3.2. Nitration of the Phl p 2 protein under UV-A irradiation and in the dark

BSA nitration results were useful to optimize the settings for the experiments with Phl p 2. Indeed, recombinant Phl p 2 was available in limited amount and the experiments with acidic nitrate solutions were performed at all pH values considered for BSA (pH = 5.6, 5.0, 3.0, 2.2, 0.5), but using only two nitrate concentrations (1:1 and 1:1000), both in the dark and under UV-A irradiation (90 W m^{-2}) for a reaction time of 30 min.

Phl p 2 is a small allergen protein released by *Phleum pratense* pollen of 10.7 kDa, containing 3 Tyr residues, all on the surface of the protein, that may be nitrated in the reaction conditions. Results of the Phl p 2 nitration experiments are summarized in Fig. 3A. Interestingly, in the experiments performed under UV-A irradiation (90 W m^{-2}) protein nitration was always observed within the entire pH range 5.6–0.5 investigated, and for both dilute (1:1000) and concentrated (1:1) nitrate solutions. These results are very similar to those presented for BSA (Section 3.1), as they also show that Phl p 2 nitration significantly increased at acidic pH and for the more concentrated (1:1) nitrate solution (see Fig. 3A).

In the dark experiments Phl p 2 nitration was only observed for the dilute 1:1000 nitrate solution and for pH ≤ 2.2 (Fig. 3A). No signal was detected in the case of the 1:1 nitrate solution at any pH. Again, these results are very similar to those reported for BSA, and they confirm the finding that protein nitration under dark conditions only occurred for the dilute nitrate solution (1:1000). In particular, nitration was observed at pH ≤ 5 for BSA and at the more acidic pH ≤ 2.2 , for Phl p 2. It should be noted that incorrect results from the method applied to evaluate protein nitration in the case of such very high 1:1 nitrate

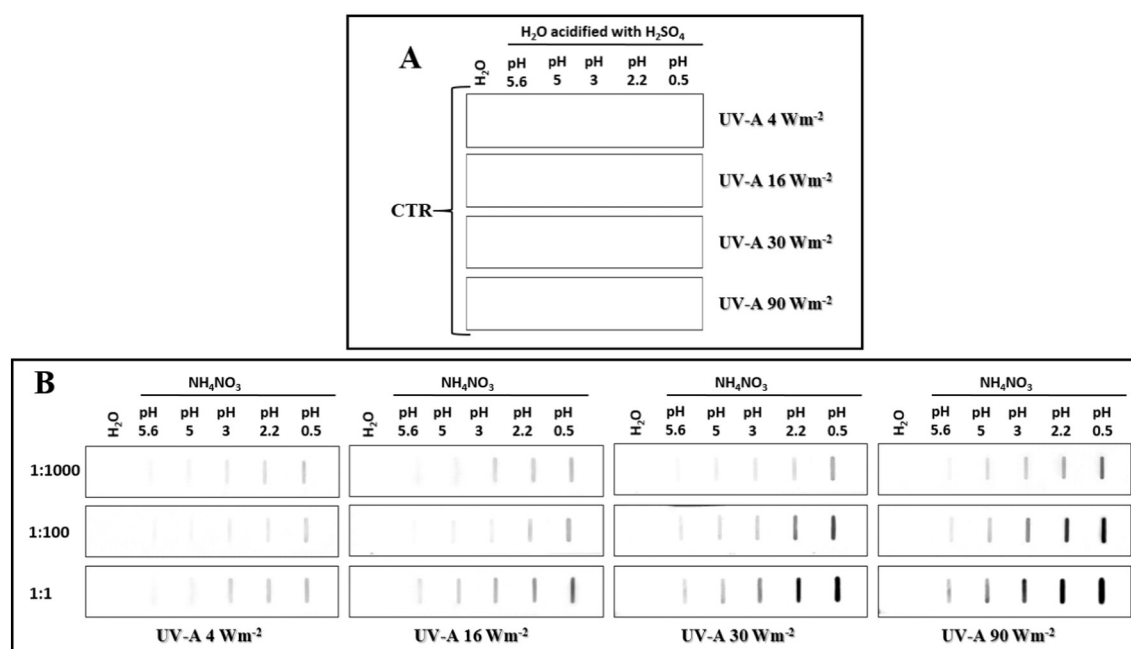


Fig. 2. BSA nitration evaluated using Slot Blot membranes probed with the antibody against 3-nitrotyrosine after treatment of the protein with NH_4NO_3 solutions at different pH (5.6, 5.0, 3.0, 2.2, 0.5), different nitrate concentrations (1:1, 1:100, 1:1000; see Table 1 for the specification of nitrate concentrations) and under exposure to UV-A light with different irradiance (4, 16, 30, 90 W m^{-2}). Samples were irradiated for 30 min. In panel A there are shown the experiments carried out with acidified water (without NH_4NO_3) as controls; in panel B there are the experiments carried out with NH_4NO_3 solutions at the different concentrations.

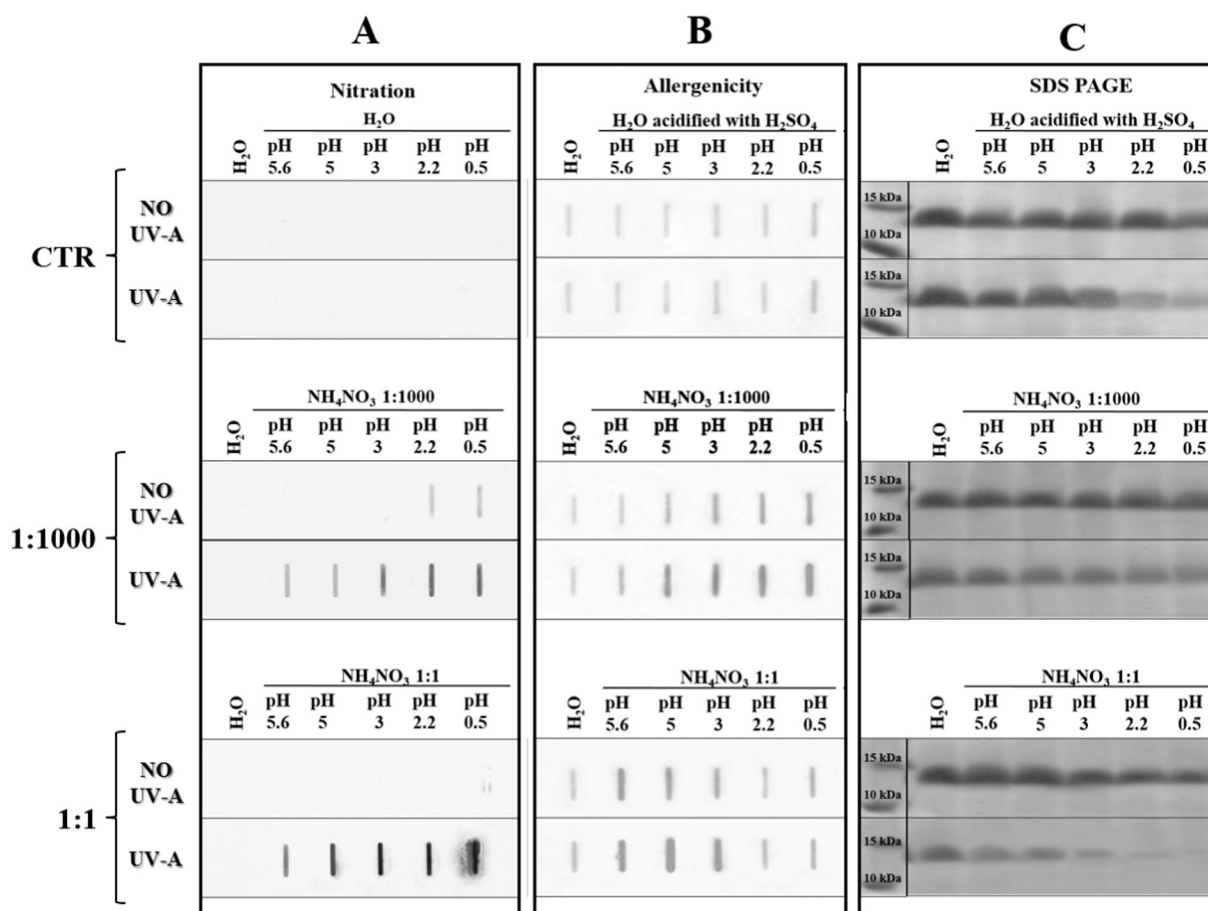


Fig. 3. Nitration, allergenicity and stability of the recombinant Phl p 2 protein treated with acidified water solutions (CTR) as controls, and with NH_4NO_3 solutions at different pH values (5.6, 5.0, 3.0, 2.2, 0.5), different nitrate concentrations (1:1, 1:1000; see Table 1 for the specification of nitrate concentrations), in the dark (no UV-A) or under exposure to UV-A light (90 W m^{-2}). Samples were irradiated for 30 min. In panel (A) there are shown Slot Blot membranes probed with the antibody against 3-nitrotyrosine, to evaluate protein nitration; in panel (B) Slot Blot membranes probed with a sera mix from grass pollen allergic patients to evaluate allergenicity, and in panel (C) SDS PAGE and gels silver staining to evaluate protein degradation.

concentration can be ruled out, since in the experiments carried out under UV-A irradiation, a large degree of protein nitration was indeed detected at high nitrate concentration.

The integrity of Phl p 2 after treatment with the acidic nitrate solutions both in the dark and under UV-A irradiation has been evaluated by the SDS-PAGE analysis reported in Fig. 3C. We note that the protein exposed to UV-A light was already partly degraded in controls (in absence of nitrate) at $\text{pH} < 3$, whereas in the dark it remained intact at any pH. When the protein was treated with acidic nitrate solutions under UV-A irradiation protein degradation was already detected at $\text{pH} = 5.6$ and significantly enhanced at lower pH and at higher nitrate concentration. Notably, for the 1:1 nitrate solution and $\text{pH} < 3$ the band corresponding to the intact protein was completely disappeared, indicating full protein degradation. Protein degradation was also observed in the dark when Phl p 2 was treated with the 1:1 nitrate solution at $\text{pH} \leq 3$, although to a much lesser extent than in the experiments carried out under UV-A irradiation. The comparison of the SDS-PAGE analysis performed for BSA and Phl p 2 clearly shows that the integrity of Phl p 2 is more affected by the reaction conditions, as protein degradation was significantly larger for Phl p 2 than for BSA in the same conditions.

3.3. Allergic response

In order to investigate the effect of Phl p 2 nitration on IgE binding, Slot Blot analysis using sera from patients mono-sensitized to grass pollen were employed. Results are reported in Fig. 3B.

As a general trend we observed that IgE reactivity of allergen Phl p 2 was enhanced with respect to controls in those cases in which the protein was nitrated. In fact, the increase of Phl p 2 allergenicity was roughly parallel to the degree of protein nitration discussed in the previous section. In particular, for the protein treated with the 1:1000 nitrate solution and exposed to UV-A light the allergenicity progressively increased by switching to more acidic pH, with a trend nearly identical to the increase in protein nitration. The same holds true by considering the enhancement of Phl p 2 allergenicity after the treatment of the protein with the 1:1000 nitrate solution in the dark. This is in agreement with literature data reporting an increase in allergenicity also for other nitrated pollen allergen, such as Bet v 1.0101 (Gruijthuisen et al., 2006).

An exception to this trend was given by the protein treated with the 1:1 nitrate solution under exposure to the UV-A light. In this case, results show that, as expected, allergenicity significantly increased by lowering the pH up to the value of 3. However, the trend was reversed in more acidic conditions, where allergenicity of Phl p 2 dropped to values similar to those of the controls (see Fig. 3B), probably due to the Phl p 2 significant degradation at these pHs, as shown by the electrophoresis of the samples (Fig. 3C). An increase of Phl p 2 allergenicity was also observed with the 1:1 nitrate solution in the dark, at pH between 5.6 and 3 despite the absence of any visible nitration signal.

In summary, Phl p 2 treated with acidic nitrate under UV-A irradiation (90 W m^{-2}) was nitrated in both dilute and concentrated solutions at all pH values (range 5.6–0.5), and nitration increased proportionally with pH decrease, as discussed in Section 3.2. Allergenicity of Phl p 2 increased in parallel with nitration in dilute 1:1000 solutions for all pH

values, and in concentrated solution 1:1 at pH 5.6, pH 5 and pH 2.2. At pHs 2.2 and 0.5, under UV-A irradiation, the allergen was significantly nitrated in the most concentrated 1:1 solution. However, the allergenicity decreased probably due to the Phl p 2 significant degradation.

4. Discussion

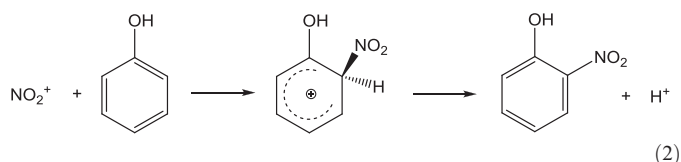
Protein nitration laboratory experiments have already been carried out by using as nitrating agents gaseous $\cdot\text{NO}_2$ and $\cdot\text{NO}_2 + \text{O}_3$ mixtures, and other (standard laboratory) nitrating reagents such as peroxonitrite (ONOO^-) and tetranitromethane (Reinmuth-Selzle et al., 2014). However, to our knowledge no experiments have been conducted by using the nitrate ion as nitrating agent, which, on the other hand, is one of the major components of PM. It can be assumed that nitrate ion containing particles come in contact with pollen proteins in the atmosphere during aerosol aging processes (e.g. NH_4NO_3 condensation on pollen grains/fragments and coagulation processes between particles), generating internally mixed particles that are commonly observed in urban air (Pöschl, 2005). As discussed in the previous sections, the liquid water phase of atmospheric particles close to the deliquescence point can feature very 'extreme' conditions, in terms of high nitrate concentration and low pH, at which the tyrosine residues in proteins may be nitrated, both in dark and/or under exposure to light, by processes similar to those already observed for activated aromatic compounds such as phenols and cresols. Indeed, the results presented in this work show that proteins such as BSA and Phl p 2 are easily nitrated by acidic nitrate aqueous solutions.

Nitration of activated aromatic molecules such as phenols and cresols has been widely investigated, and we can refer to these studies to get a better understanding of the nitration mechanisms of the tyrosine amino acid in proteins. Nitration of such aromatic compounds can occur in aqueous solutions in the dark and under irradiation following two distinct processes: the well-known aromatic electrophilic substitution mechanism (AES), and a photochemical induced radical mechanism, respectively.

The electrophilic substitution reaction is known to occur on activated aromatic rings in the presence of nitrate ions and very acidic conditions, such that the nitronium ion (NO_2^+) can be formed:



NO_2^+ can then react with the activated aromatic compound through a bimolecular mechanism yielding to final ortho (or para) nitrated compounds:



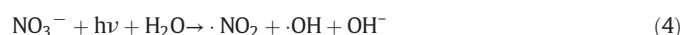
In the literature, many studies are present that describe the mechanism of phenol nitration *via* AES because of the relevance of this reaction for organic synthesis and industrial applications (March, 1988). This reaction has been only rarely considered for the nitration of aromatic compounds under environmental conditions since it is assumed that such extreme acidic conditions are not achieved. However, in the liquid water phase of atmospheric particles, conditions characterized by very low pHs and high nitrate concentrations can be attained.

The results presented in this work show that protein nitration indeed occurs in the dark under the most acidic conditions considered (pH = 3.3 for BSA, and pH $\leq$ 2.2 for Phl p 2, in 1:1000 solutions, 30 min). This finding agrees with the results reported by Vione et al. (2001) concerning the nitration of phenol by acidic nitrate aqueous solutions in the dark. The amount of nitrophenols formed at pH = 1 by the

AES mechanism (in the dark) was reported to be about the 30% of the total nitrophenols generated under irradiation (*vide infra*). Nitrophenols generated by the AES mechanism were not observed for pH higher than 1.

Notably, we found an inverse relationship between the nitrate concentration and the degree of protein nitration in the dark, as the signal corresponding to protein nitration was less intense when the concentration increased from 1:1000 to 1:100 for the BSA (see Fig. 1), and completely disappeared by further increasing the nitrate concentration to 1:1 (for both BSA and Phl p 2, see Fig. 1 and Fig. 3). In this context, it is worth noting that, in the solution 1:1, the ammonium nitrate is extremely concentrated ($>13 \text{ mol kg}^{-1}$) (Table 1), and the amount of water in the liquid phase is very low (0.5–0.6 mol fraction; Table S1). In these very peculiar conditions, protein hydration might be limited, whereas it has been observed that protein hydration is necessary for the nitration reaction to occur (Shiraiwa et al., 2012b).

Exposure to UV-A light has a dramatic effect on the degree of protein nitration, as illustrated in Fig. 1 and Fig. 3. The photolysis of activated aromatic compounds in aqueous solution should occur through a radical mechanism initiated, upon irradiation at $\lambda > 280 \text{ nm}$, by the photolysis of nitrate through the following two processes (Mack and Bolton, 1999; Vione et al., 2003):



The photogenerated $\cdot\text{NO}_2$ is the main nitrating agent in the case of phenolic compounds in aqueous solution (De Laurentis et al., 2013), through a mechanism that should proceed by the H-abstraction of the phenolic oxygen by $\cdot\text{NO}_2$ to give HNO_2 and the phenoxy radical in the first step, followed by the addition of another NO_2 to finally yield the nitrophenol (Bedini et al., 2012).

In this work we have shown that the degree of protein nitration under UV-A irradiation, both for the BSA and Phl p 2 proteins, significantly increased by (i) lowering the pH, (ii) increasing the concentration of nitrate and, (iii) raising the irradiance of the lamp. The effect of the pH observed in protein nitration is similar to that reported in previous works for the nitration of phenol upon nitrate photolysis. The enhanced effect of the pH on the formation of nitrophenols was explained by considering two mechanisms; (i) the protonation of NO_2^- generated by reaction (3) to form HNO_2 (Vione et al., 2001), which then directly reacts with phenol, and (ii) the H^+ -assisted heterolytic cleavage of the N–N bond of N_2O_4 promoted at low pH (Machado and Boule, 1995)



producing the nitronium ion (NO_2^+) as electrophilic nitrating agent. The nitration by the AES mechanism in the more acidic conditions could also significantly contribute to the protein nitration, as observed by Vione et al. (2001) for the nitration of phenol. In addition, protein denaturation and degradation observed at low pH should make accessible to nitration tyrosine residues, which are in the interior of the folded proteins, further accounting for the increase of the degree of protein nitration in such acidic environment.

In some of the experimental conditions investigated in this study proteins can degrade or aggregate, possibly altering their allergenicity due to other modifications than nitration. The results obtained from the SDS Page analysis show that in the dark both BSA and Phl p 2 remained intact within the range of pHs and nitrate concentrations investigated, with the exception of Phl p 2 when treated with the 1:1 nitrate solution, for which a partial degradation was observed for pH < 5 . On the other hand, in the experiments carried out under

irradiation a significant protein degradation was detected, which was gradually enhanced by lowering the pH and increasing the nitrate concentration. In particular, for the most concentrated 1:1 nitrate solution, considerable BSA and Phl p 2 degradation was already observed at pH = 5, and it was almost complete at pH = 0.5 (see Fig. 3 and Fig. S1). It is interesting to note that significant degradation of the proteins only occurs due to the combined effects of acidic pH, light irradiation and high nitrate concentration. This effect can be explained by considering the formation of ROS such as the hydroxyl radical as by-products in nitrate photolysis, which are well known to produce oxidative damage and fragmentation of proteins (Xu and Chance, 2007). In this respect, the nitrate ion appears to be a very important ingredient of the recipe, with a role that may deserve more attention in studies of the photodegradation of proteins under environmental conditions.

The ultimate goal of this study is to get a deeper insight into the allergenicity of pollen proteins such as Phl p 2, after treatment with nitrate solutions simulating conditions expected in the atmospheric aerosol liquid water phase. As discussed above, modification of the protein can be due to protein nitration and/or protein degradation. The Slot Blot analysis reported in Fig. 4B allows evaluating, irrespective of the type of modification, the allergenicity of Phl p 2, through the binding of the treated protein with the corresponding IgE contained in sera from patients mono-sensitized to grass pollen. Interestingly, the observed trends are parallel to those discussed for the protein nitration, at least in those conditions for which Phl p 2 is not significantly degraded. Namely, in the dark the allergenicity was slightly enhanced with respect to controls after the treatment of the protein with the 1:1000 nitrate solution at low pH, whereas under irradiation the allergenicity was considerably enhanced with respect to controls by lowering the pH and by increasing the nitrate concentration. These results suggest that the 'intact' nitrated protein enhances its allergenic potential and is expected to increase the allergic response. The peculiar trend observed for the 1:1 nitrate solution under irradiation, showing a maximum in the allergic response at pH = 3 and a significant reduction at the lower pHs, can be explained by considering the complete degradation of the protein in these conditions, that evidently affects the recognition by the IgE. It is worth noting that the aim of our laboratory study was to investigate the specific nitration reaction of protein with nitrate ion in acidic water solutions simulating the conditions of air-borne liquid particles, but, due to the complex chemical composition of atmospheric PM, it is expected that various other compounds/pollutants would also be relevant in inducing protein post-translational modifications in atmospheric particles, eventually enhancing allergenicity (D'Amato et al., 2007).

5. Conclusion

Pollen aeroallergens are widespread, and they can remain for a long time in air, mainly associated with fine atmospheric PM. A lot of study is still needed to get a better understanding of the protein reactivity in the atmosphere, even though several studies have appeared reporting that protein nitration in polluted air is one of the most important posttranslational modifications of allergenic proteins that would explain the enhancement of allergic diseases.

In this study, we suggest the potential role of the nitrate ion, which is particularly abundant in polluted air, as responsible for protein nitration occurring in the bulk liquid water phase of atmospheric particles, under acidic conditions. We found that protein nitration can occur in acidic nitrate solutions in the dark only at very low pH (pH < 3 for BSA; pH < 2.2 for Phl p 2) in diluted nitrate solutions. Protein nitration was much more relevant under UV-A irradiation, where it was observed for both diluted and concentrated (close to saturation) nitrate solutions, in the pH range 5.6–0.5, due to a photolysis mechanism greatly strengthened by low pH.

Nitration promoted by these conditions enhances protein allergenicity modifying its immunological properties. However, very acidic pH

and UV-A irradiation seems to cause protein degradation, which, on the contrary, leads to a decrease in the allergenic response. Further studies will be needed to fully understand both protein nitration and protein degradation in air, and how they can combine in altering the allergic response to the allergenic protein present in the atmospheric environment.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2016.09.041>.

References

- Bacsi, A., Choudhury, B.K., Dharajiya, N., Sur, S., Boldogh, I., 2006. Subpollen particles: carriers of allergenic proteins and oxidases. *J Allergy Clin Immunol* 118 (4), 844–850.
- Bedini, A., Maurino, V., Minero, C., Vione, D., 2012. Theoretical and experimental evidence of the photolysis pathway of phenol and 4-chlorophenol: a mechanistic study of environmental significance. *Photochem. Photobiol. Sci.* 11, 418–424.
- Behera, S.N., Betha, R., Liu, P., Balasubramanian, R., 2013. A study of diurnal variations of PM_{2.5} acidity and related chemical species using a new thermodynamic equilibrium model. *Sci. Total Environ.* 452–53, 286–295.
- Bencs, L., Ravindra, K., de Hoog, J., Rasoazananany, E.O., Deutsch, F., Bleux, N., Berghmans, P., Roekens, E., Krata, A., van Grieken, R., 2008. Mass and ionic composition of atmospheric fine particles over Belgium and their relation with gaseous air pollutants. *J. Environ. Monit.* 10, 1148–1157.
- Blando, J., Bielory, L., Nguyen, V., Diaz, R., Jeng, H.A., 2012. Anthropogenic climate change and allergic diseases. *Atmosfera* 3, 200–212.
- Breiteneder, H., Ebner, C., 2000. Molecular and biochemical classification of plant derived food allergens. *J Allergy Clin Immunol* 106, 27–36.
- Buters, T.M.J., Kasche, A., Weichenmeier, I., Schober, W., Klaus, S., Traill-Hoffmann, C., et al., 2008. Year to year variation in release of Bet v 1 allergen from birch pollen: evidence for geographical differences between west and south Germany. *Int. Arch. Allergy Immunol.* 145, 122–130.
- Buters, T.M.J., Weichenmeier, I., Ochs, S., Pusch, G., Kreyling, W., Boere, A.J.F., et al., 2010. The allergen Bet v 1 in fractions of ambient air deviates from birch pollen counts. *Allergy* 67 (7), 850–858.
- Casati, M., Rovelli, G., D'Angelo, L., MG, P., Sangiorgi, G., Bolzacchini, E., et al., 2015. Experimental measurements of particulate matter deliquescence and crystallization relative humidity: application in heritage climatology. *Aerosol Air Qual. Res.* 15 (2), 399–409.
- Choi, G.S., Kim, J.H., Lee, H.N., Sung, J.M., Lee, J.W., Park, H.S., 2009. Occupational asthma caused by inhalation of bovine serum albumin powder. *Allergy Asthma Immun* 1, 45–47.
- Clegg, S.L., Brimblecombe, P., Wexle, A.S., 1998. A thermodynamic model of the system H^+ , NH_4^+ , SO_4^{2-} , NO_3^- , H_2O at tropospheric temperatures. *J Phys Chem A* 102, 2137–2154.
- D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., et al., 2007. Allergic pollen and pollen allergy in Europe. *Allergy* 62 (9), 976–990.
- D'Angelo, L., Rovelli, G., Casati, M., Sangiorgi, G., Perrone, M.G., Bolzacchini, E., et al., 2016. Seasonal behavior of PM_{2.5} deliquescence, crystallization, and hygroscopic growth in the Po Valley (Milan): implications for remote sensing applications. *Atmos. Res.* 176–7, 87–95.
- De Laurentis, E., Socorro, J., Vione, D., Quivet, E., Brigante, M., Mailhot, G., et al., 2013. Phototransformation of 4-phenoxy sensitized by 4-carboxybenzophenone: evidence of new photochemical pathways in the bulk aqueous phase and on the surface of aerosol deliquescent particles. *Atmos. Environ.* 81, 569–578.
- De Linares, C., Nieto Lugilde, D., Alba, F., Díaz de la Guardia, C., Galán, C., Trigo, M.M., 2007. Detection of airborne allergen (Ole e 1) in relation to *Olea europaea* pollen in S Spain. *Clin. Exp. Allergy* 37, 125–132.
- De Linares, C., Díaz de la Guardia, C., Nieto Lugilde, D., Alba, F., 2010. Airborne study of grass allergen (lol p 1) in different-sized particles. *Int. Arch. Allergy Immunol.* 152, 49–57.
- Farkhondeh R and Ahmad M. The effects of air pollution on cellular material release, allergenicity and allergens proteins of three ornamental plants. In: *Advanced Topics in Environmental Health and Air Pollution Case Studies*, Chapter 25. Book edited by Anca Maria Moldoveanu. Publisher: InTech; 2011. ISBN 978-953-307-525-9
- Fernández-González, D., González-Parrado, Z., Vega-Maray, A.M., Valencia Barrera, R.M., Camazón-Izquierdo, B., De Nuntiis, P., et al., 2010. *Platanus* pollen allergen, Pla a 1:

- quantification in the atmosphere and influence on a sensitizing population. *Clin. Exp. Allergy* 40, 1701–1708.
- Franze, T., Weller, M.G., Niessner, R., Pöschl, U., 2005. Protein nitration by polluted air. *Environ. Sci. Technol.* 39, 1673–1678.
- Grujthuijsen, Y.K., Grieshuber, I., Stockinger, A., Tischler, U., Fehrenbach, T., Weller, M.G., et al., 2006. Nitration enhances the allergenic potential of proteins. *Int. Arch. Allergy Immunol.* 141, 265–275.
- Lide DR, Haynes WM (Eds) CRC Handbook of Chemistry and Physics 91st ed., Publisher: CRC Press; 2010. ISBN 1-4398-2077
- Lüttke, J., Scheer, V., Levsen, K., Wünsch, G., Cape, J.N., Hargreaves, K.J., et al., 1997. Occurrence and formation of nitrated phenols in and out of cloud. *Atmos. Environ.* 31 (16), 2637–2648.
- Machado, F., Boule, P., 1995. Photonitration and photonitrosation of phenolic derivatives induced in aqueous solution by excitation of nitrite and nitrate ions. *J. Photochem. Photobiol. A Chem.* 86, 73–80.
- Mack, J., Bolton, J.R., 1999. Photochemistry of nitrite and nitrate in aqueous solution: a review. *J. Photochem. Photobiol. A* 128, 1–13.
- March, J., 1998. *Advanced Organic Chemistry: Reactions, Mechanisms, and Structures*. 3rd Ed. John Wiley & Sons, New York, p. 447.
- Miguel, A.G., Taylor, P.E., House J, Glovsky, M.M., Flagan, R.C., 2006. Meteorological influences on respirable fragment release from Chinese elm pollen. *Aerosol Sci. Technol.* 40 (9), 690–696.
- Perrone, M.G., Gualtieri, M., Ferrero, L., Lo Porto, C., Udisti, R., Bolzacchini, E., et al., 2010. Seasonal variations in chemical composition and in vitro biological effects of fine PM from Milan. *Chemosphere* 78, 1368–1377.
- Perrone, M.G., Larsen, B.R., Ferrero, L., Sangiorgi, G., De Gennaro, G., Udisti, R., et al., 2012. *Sci. Total Environ.* 414, 343–355.
- Pöschl, U., 2005. Atmospheric aerosols: composition, transformation, climate and health effects. *Angew. Chem. Int. Ed.* 44, 7520–7540.
- Putaud, J.P., Van Dingenen, R., Alastuey, A., Bauer, H., Birmili, W., Cyrys, J., et al., 2010. A European aerosol phenomenology – 3.: physical and chemical characteristics of particulate matter from 60 rural, urban, and kerbside sites across Europe. *Atmos. Environ.* 44, 1308–1320.
- Radauer, C., Breiteneder, H., 2006. Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. *J. Allergy Clin Immunol* 117 (1), 141–147.
- Reinmuth-Selzle, K., Ackaert, C., Kampf, C.J., Samonig, M., Shiraiwa, M., Kofler, S., et al., 2014. Nitration of the birch pollen allergen Bet v 1.0101: efficiency and site-selectivity of liquid and gaseous nitrating agents. *J. Proteome Res.* 13, 1570–1577.
- Seckmeyer, G., Pissulla, D., Glandorf, M., Henriques, D., Johnen, B., Webb, A., et al., 2008. Variability of UV irradiance in Europe. *Photochem. Photobiol.* 84, 172–179.
- Seinfeld, J.H., Pandis, S.N., 2006. *Atmospheric chemistry and physics. From Air Pollution to Climate Change*, Second edition A Wiley-Interscience Publication.
- Shiraiwa, M., Selzle, K., Pöschl, U., 2012a. Hazardous components and health effects of atmospheric aerosol particles: reactive oxygen species, soot, polycyclic aromatic compounds and allergenic proteins. *Free Radic. Res.* 46 (8), 927–939.
- Shiraiwa, M., Selzle, K., Yang, H., Sosedova, Y., Ammann, M., Pöschl, U., 2012b. Multiphase chemical kinetics of the nitration of aerosolized protein by ozone and nitrogen dioxide. *Environ. Sci. Technol.* 46, 6672–6680.
- Sofiev M, Bergmann KC. Allergenic Pollen. A Review of the Production, Release, Distribution and Health Impacts. Editors: Sofiev M, Bergmann KK(Eds.). Publisher: Springer Netherlands; 2013. DOI: <http://dx.doi.org/10.1007/978-94-007-4881-1>
- Squizzato, S., Masiol, M., Brunelli, A., Pistollato, S., Tarabotti, E., Rampazzo, G., et al., 2013. Factors determining the formation of secondary inorganic aerosol: a case study in the Po Valley (Italy). *Atmos. Chem. Phys.* 13, 1927–1939.
- Taylor, P.E., Flagan, R.C., Miguel, A.G., Valenta, R., Glovsky, M.M., 2004. Birch pollen rupture and the release of aerosols of respirable allergens. *Clin. Exp. Allergy* 34, 1591–1596.
- Vione, D., Maurino, V., Minero, C., Vincenti, M., Pellizzetti, E., 2001. Formation of nitrophenols upon UV irradiation of phenol and nitrate in aqueous solutions and in TiO₂ aqueous suspensions. *Chemosphere* 44 (2), 237–248.
- Vione, D., Maurino, V., Minero, C., Borghesi, D., Lucchiari, M., Pelizzetti, E., 2003. New processes in the environmental chemistry of nitrite. 2. The role of hydrogen peroxide. *Environ. Sci. Technol.* 37, 4635–4641.
- Vione, D., Maurino, V., Minero, C., Pelizzetti, E., Harrison, M.A.J., Olariu, R.I., et al., 2006. Photochemical reactions in the tropospheric aqueous phase and on particulate matter. *Chem. Soc. Rev.* 35, 441–453.
- Voltolini, S., Spigno, F., Cioè, A., Cagnati, P., Bignardi, D., Minale, P., 2013. Bovine serum albumin: a double allergy risk. *Eur Ann Allergy Clin Immunol* 45 (4), 144–147.
- Walcher, W., Franze, T., Weller, M.G., Pöschl, U., Huber, C.G., 2003. Liquid- and gas-phase nitration of bovine serum albumin studied by LC-MS and LC-MS/MS using monolithic columns. *J. Proteome Res.* 2, 534–542.
- Wexler AS and Clegg SL Atmospheric aerosol models for systems including the ions H⁺, NH₄⁺, Na⁺, SO₄²⁻, NO₃⁻, Cl⁻, Br⁻ and H₂O. *J. Geophys. Res.* 2002; 107 No. D14: art no. 4207
- Xu, G., Chance, M.R., 2007. Hydroxyl radical mediated modification of proteins as probes for structural proteomics. *Chem. Rev.* 107, 3514–3543.
- Zhang, Y., Yang, H., Pöschl, U., 2011. Analysis of nitrated proteins and tryptic peptides by HPLC-chip-MS/MS: site-specific quantification, nitration degree, and reactivity of tyrosine residues. *Anal. Bioanal. Chem.* 399 (1), 459–471.

Nitration of Pollen Aeroallergens by Nitrate Ion in Conditions Simulating the Liquid Water Phase of Atmospheric Particles

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Supplementary Material

Atmospheric conditions	INPUT DATA			OUTPUT DATA								
	atm ion conc			ion conc in the liquid water phase								
	$\mu\text{g m}^{-3}$	$\mu\text{eq m}^{-3}$		MW (g mol^{-1})	mol kg ⁻¹ H ₂ O	mole fraction	mole fraction activity coeff (fj *)	mole activity coeff γ	mol L ⁻¹ sol	g L ⁻¹ sol		
Complete neutralization	NO ₃ ⁻ (atm)	0.80	0.013	NO ₃ ⁻ (aq)	62	1.34E+01	1.47E-01	2.68E-01	1.63E-01	6.98E+00	4.33E+02	
	SO ₄ ²⁻ (atm)	3.58	0.075	SO ₄ ²⁻ (aq)	96	3.01E+00	3.30E-02	4.36E-02	2.65E-02	1.57E+00	1.51E+02	
	NH ₄ ⁺ (atm)	1.58	0.088	NH ₄ ⁺ (aq)	18	1.94E+01	2.13E-01	3.12E-01	1.90E-01	1.01E+01	1.82E+02	
	NR	1.000		HSO ₄ ⁻ (aq)	97	2.22E-04	2.44E-06	4.52E-01	2.75E-01	1.16E-04	1.13E-02	
				NH ₃ (aq)	17	2.32E-04	2.54E-06	1.65E+00	1.00E+00	1.21E-04	2.06E-03	
				OH ⁻ (aq)	17	2.57E-11	2.81E-13	4.77E+01	2.90E+01	1.34E-11	2.28E-10	
				H ₂ O (aq)	18	5.55E+01	6.08E-01	1.11E+00	6.76E-01	2.90E+01	5.22E+02	
				H ⁺ (aq)	1	9.60E-06	1.05E-07	1.43E+00	8.68E-01	5.02E-06	5.02E-06	
				pH					5.36			
				DRH (@ 25° C)					68%			
				density liquid phase (g cm ⁻³)					1.29			
Very low ammonium deficit (0.2 %)	NO ₃ ⁻ (atm)	0.80	0.013	NO ₃ ⁻ (aq)	62	1.31E+01	1.43E-01	2.65E-01	1.62E-01	6.84E+00	4.24E+02	
	SO ₄ ²⁻ (atm)	3.58	0.075	SO ₄ ²⁻ (aq)	96	3.05E+00	3.35E-02	4.14E-02	2.52E-02	1.60E+00	1.53E+02	
	NH ₄ ⁺ (atm)	1.57	0.087	NH ₄ ⁺ (aq)	18	1.93E+01	2.12E-01	3.18E-01	1.94E-01	1.01E+01	1.82E+02	
	NR	0.998		HSO ₄ ⁻ (aq)	97	1.64E-01	1.80E-03	4.52E-01	2.76E-01	8.57E-02	8.32E+00	
				NH ₃ (aq)	17	3.08E-07	3.38E-09	1.64E+00	1.00E+00	1.61E-07	2.74E-06	
				OH ⁻ (aq)	17	3.45E-14	3.79E-16	4.62E+01	2.82E+01	1.81E-14	3.07E-13	
				H ₂ O (aq)	18	5.55E+01	6.09E-01	1.11E+00	6.76E-01	2.90E+01	5.23E+02	
				H ⁺ (aq)	1	7.32E-03	8.03E-05	1.43E+00	8.72E-01	3.83E-03	3.83E-03	
				pH					2.48			
				DRH (@ 25° C)					67%			
				density liquid phase (g cm ⁻³)					1.29			
10 % ammonium deficit	NO ₃ ⁻ (atm)	0.80	0.013	NO ₃ ⁻ (aq)	62	1.39E+01	1.29E-01	1.66E-01	8.55E-02	5.73E+00	3.55E+02	
	SO ₄ ²⁻ (atm)	3.58	0.075	SO ₄ ²⁻ (aq)	96	3.78E+00	3.51E-02	1.18E-02	6.10E-03	1.56E+00	1.50E+02	
	NH ₄ ⁺ (atm)	1.42	0.079	NH ₄ ⁺ (aq)	18	2.77E+01	2.57E-01	4.81E-01	2.48E-01	1.14E+01	2.05E+02	
	NR	0.900		HSO ₄ ⁻ (aq)	97	6.58E+00	6.10E-02	5.47E-01	2.81E-01	2.71E+00	2.63E+02	
				NH ₃ (aq)	17	4.12E-09	3.82E-11	1.94E+00	1.00E+00	1.70E-09	2.88E-08	
				OH ⁻ (aq)	17	3.55E-17	3.29E-19	3.30E+02	1.70E+02	1.46E-17	2.49E-16	
				H ₂ O (aq)	18	5.55E+01	5.15E-01	1.11E+00	5.74E-01	2.29E+01	4.12E+02	
				H ⁺ (aq)	1	3.68E-01	3.42E-03	4.61E+00	2.37E+00	1.52E-01	1.52E-01	
				pH					0.44			
				DRH (@ 25° C)					57%			
				density liquid phase (g cm ⁻³)					1.39			

Table S1. Ion concentrations in the liquid water phase of atmospheric PM and acidity (pH) at Deliquescence Relative Humidity (DRH), when aerosol water content is minimum, simulated by E-AIM model. As input data we referred to the atmospheric concentrations of major anions NO₃⁻ and SO₄²⁻ ($\mu\text{g m}^{-3}$; average summer values for PM_{2.5} samples in Milan, North Italy) and three different NH₄⁺ neutralization conditions (NR= neutralization ratio): complete neutralization (NR=1.000); very low ammonium deficit, 0.2 % (NR=0.998); 10 % ammonium deficit (NR=0.900).

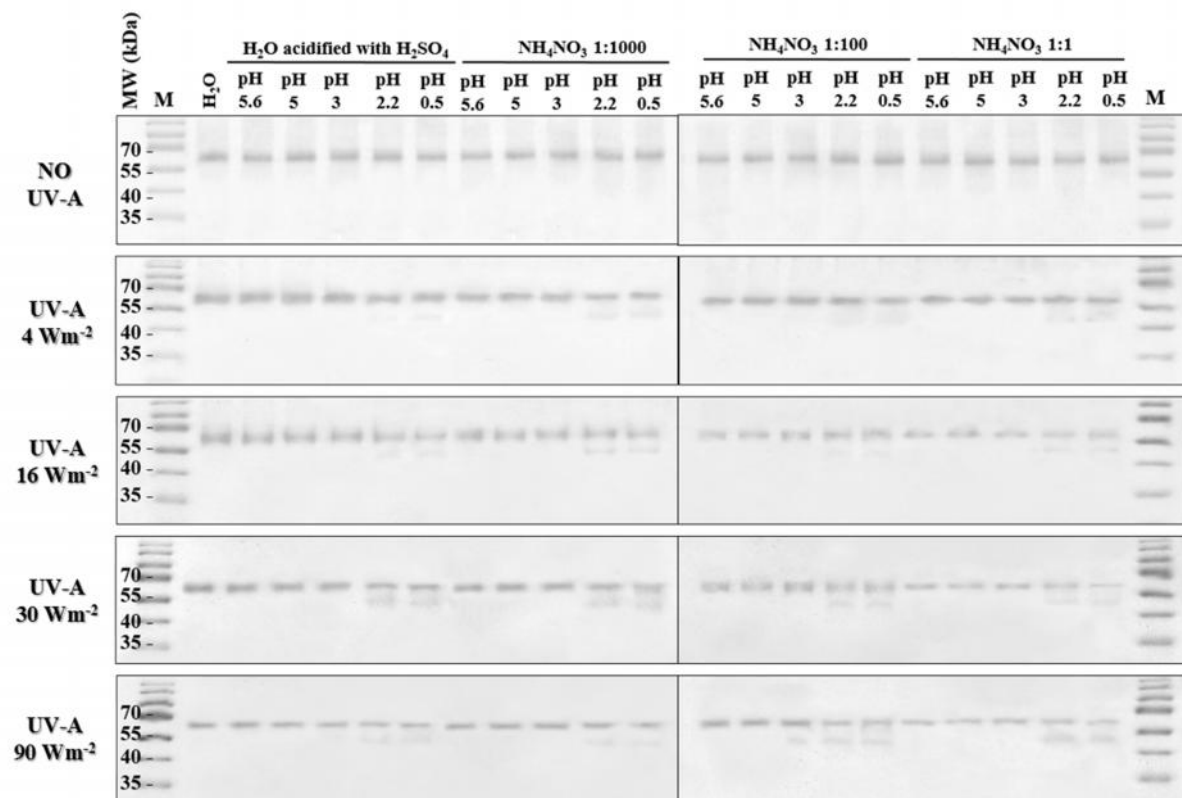


Fig. S1. Qualitative BSA analysis by SDS-PAGE and silver nitrate staining after protein treatment under power increasing UV-A irradiation (4, 16, 30, 90 W m⁻²) for 30 minutes, with acidified water and different NH₄NO₃ concentrations (1:1, 1:100, 1:1000; see Table 1 for the specification of concentrations) at decreasing pH (5.6 to 0.5). M: protein ladder.