



Allergenicity of recombinant *Humulus japonicus* pollen allergen 1 after combined exposure to ozone and nitrogen dioxide[☆]

Qiang Hong^{a, b, 1}, Shumin Zhou^{b, 1}, Hui Zhao^a, Jiaxian Peng^a, Yang Li^b, Yu Shang^b, Minghong Wu^a, Wei Zhang^{b, **}, Senlin Lu^{a, *}, Shuijun Li^c, Shen Yu^c, Weiqian Wang^d, Qingyue Wang^{d, ***}

^a School of Environmental and Chemical Engineering, Shanghai University, Shanghai 200444, China

^b School of Life Sciences, Shanghai University, Shanghai 200444, China

^c Shanghai Xuhui Center Hospital, Shanghai 200031, China

^d School of Science and Engineering, Saitama University, Saitama 338-8570, Japan

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ABSTRACT

Ozone (O₃) and nitrogen dioxide (NO₂) are thought to play primary roles in aggravating air pollution-induced health problems. However, the effects of joint O₃/NO₂ on the allergenicity of pollen allergens are unclear. *Humulus japonicus* pollen allergen 1 (Hum j1) is a profilin protein that causes widespread pollinosis in eastern Asia. In order to study the effects of combined O₃/NO₂ on the allergenicity of Hum j1, tandem six-histidine peptide tag (His6)-fused recombinant Hum j1 (rHum j1) was expressed in a prokaryotic system and purified through His6 affinity chromatography. The purified rHum j1 was used to immunize SD rats. Rat sera with high titers of IgG and IgE antibodies against rHum j1 were used for allergenicity quantification. The rHum j1 was exposed to O₃/NO₂, and changes in allergenicity of the exposed rHum j1 were assayed using the immunized rat antibodies. Tandem LC-MS/LC (liquid chromatography-mass spectrometer/liquid chromatography spectrometer) chromatography and UV and circular dichroism (CD) spectroscopy were used to study the structural changes in rHum j1. Our data demonstrated that a novel disulfide bond between the sulfhydryl groups of two neighboring cysteine molecules was formed after the rHum j1 exposure to joint O₃/NO₂, and therefore IgE-binding affinity was increased and the allergenicity was reinforced. Our results provided clues to elucidate the mechanism behind air pollution-induced increase in pollinosis prevalence.

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1. Introduction

With the rapid economic growth in China, high levels of anthropogenic pollutants, including particulate matters, NO_x, and O₃, have been reported in urban atmosphere; a corresponding increase in the incidence of allergenic diseases has also been reported (Riediker et al., 2001; Kan et al., 2009; Wong et al., 2013; Platt-Mills, 2015; Bunne et al., 2017; Ribeiro et al., 2014). In addition,

an increasing number of pollen allergens are being identified, such as ragweed allergen Amb a1, mugwort allergen Art v4, birch pollen allergen Bet v1, cedar pollen allergen Cry j2, plane tree allergens Pla a1,a2,a3, and *Humulus* allergen Hum j1 (Smole et al., 2010; Acevedo et al., 2016; Offermann et al., 2016; Zhao et al., 2016). *Humulus japonicus* growth is widespread in eastern Asia, and the *Humulus* pollen is one of the primary causes of pollinosis in the fall season (Li and Wang, 1986; Park et al., 1999; Segawa et al., 2007; Sun et al., 2017; Tao and He, 2005; Jeong et al., 2013). The outer wall of pollen grains can be broken by rainwater, or high humidity, causing the release of its intracellular contents, including allergenic proteins (Bacsi et al., 2006; Wang et al., 2012; Pazmandi et al., 2012; Silva et al., 2015). These allergenic proteins, which are inhaled into the human body from the air, can cause allergic reactions. Previous studies have shown that the incidence of respiratory allergies, including asthma, is associated with urban air pollution, a potential

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^{*} Corresponding author.

^{**} Corresponding author.

^{***} Corresponding author.

E-mail addresses: zhw0627@staff.shu.edu.cn (W. Zhang), senlinlv@staff.shu.edu.cn (S. Lu), seiyo@mail.saitama-u.ac.jp (Q. Wang).

¹ Authors equally contributed to the work.

driver for the increasing prevalence of allergic diseases (Ring et al., 2001; Lang-Yona et al., 2016). Vehicle emissions, one of the main sources of urban air pollution, contain a high concentration of nitrogen oxides, sulfates, and particulate matters (Beckerman et al., 2012; Deville Cavellin et al., 2016). Air pollutants can affect the human immune system by increasing the incidence of airway inflammation and allergic reactions (Chuang et al., 2007; Arantes-Costa et al., 2014). In addition, some pollutants can alter the chemical characteristics of airborne allergens through post-translational modification (PTM) during their transport in the atmosphere or affect the transcription of allergenic proteins in pollens, thus increasing the incidence of pollen allergies (Silva et al., 2015; Zhao et al., 2017). For example, nitration of birch pollen allergen, Bet v1.0101, by mixtures of nitrogen dioxide (NO_2) and O_3 not only altered the transcriptome of Bet v1, but also affected the molecular structure and immunogenicity of the protein, thus aggravating the allergic reaction (Franze et al., 2003; Gruijthuisen et al., 2006; Ackaert et al., 2014; Kampf et al., 2015). Ragweed pollen allergen, Amb, showed differential transcript expression when exposed to $\text{NO}_2 + \text{O}_3$ (Zhao et al., 2017). Furthermore, the expression levels of *Platanus orientalis* pollen allergens (Pla 1, 2, 3) were significantly affected by traffic pollutants (Ribeiro et al., 2017).

NO_2 and O_3 are the major gaseous pollutants in the troposphere in highly urbanized areas. Ground-level O_3 is a secondary pollutant, which is produced by photochemical reactions between volatile organic compounds and nitrogen oxides during the combustion of fossil fuels (Frank and Ernst, 2016). NO_2 is the main pollutant in the industrial combustion process and in automobile exhausts (Deville Cavellin et al., 2016; Frank and Ernst, 2016). NO_x can not only influence the morphology of pollens, but also increase the aggression of pollen allergens (Ring et al., 2001; Zhao et al., 2016). Pollens exposed to NO_2 and O_3 can trigger higher levels of IgE in allergic individuals (Gilmour, 1995; Weir et al., 2013; Cuinica et al., 2014; Ribeiro et al., 2014, 2017; Silva et al., 2015). Although air pollutants have been reported to modulate many grass pollen allergens (Behrendt et al., 1997; Teran et al., 2009; Zhao et al., 2016, 2017), the effect of NO_2 and O_3 (Hereinafter referred as to $\text{NO}_2 + \text{O}_3$) on *Humulus japonicus* pollen allergen 1 (Hum j1) has not been investigated.

Allergenic proteins released from pollens are complex. Therefore, we expressed and purified a recombinant tandem 6-histidine short peptide tag-fused Hum j1 (rHum j1) in a prokaryotic expression system using tag affinity chromatography. Recombinant protein technology is an important biological method for the structural and functional study of proteins. The artificial expression of target proteins can not only enrich the micro-protein in the organism, but also effectively separate and purify it from complex components. Recombinant proteins have been widely used for studying biological characteristics, structures, and functions of allergenic pollen proteins such as Pla a1, Che a1, and FPH4 (Vahedi et al., 2011; Pichler et al., 2014; Liu et al., 2015).

Previous studies have identified a number of clues to the relationship between air pollution and pollen allergy, including the effects of pollutants on the integrity of pollen, the amount of allergens and the allergenic potency of allergenic proteins. But the molecular mechanism of allergenicity is not very clear. In this study, we aimed to investigate the effects of combined NO_2 and O_3 on the allergenicity of the rHum j1 and to reveal structural changes of the allergenic protein (Fig. S1).

2. Methods

2.1. Plant materials

Pollens were collected from mature *Humulus* inflorescence and

stored in eppendorf tubes at -20°C after screening with a $40\text{-}\mu\text{m}$ mesh. *Arabidopsis thaliana* was grown on Murashige-Skoog (MS) medium (Solarbio Life Sciences, China) and cultured at 23°C for 2 weeks. Then the seedlings were collected and the total protein was extracted by plant total protein extraction kit (Sigma-Aldrich, China). SD rats and Balb/c mice were purchased from Sliker Centre of Experimental Animals (Shanghai, China) and housed under pathogen-free conditions within the animal care facility in the lab animal room of the Shanghai University. All animal experiments were approved by the Animal Experiments Committee of the School of Life Science, Shanghai University. The *Escherichia coli* Rosetta strain was stored by our research group.

2.2. Recombinant Hum j1-His protein expressing constructs

The CDS of the rHum j1 was amplified by PCR using the following oligonucleotide primers: 5'-TGCCATGGCTAAGAACTGTCAGCATCACAACA-3' and 5'-CCAAGCTTGTGAGAGCCACTTTGATGATCGATC-3'. It was then double-digested with *Nco*I and *Hind*III, ligated to pET-30a, and transformed into the *E. coli* Rosetta strain.

2.3. Protein purification

The pET30a-rHum j1-harboring Rosetta stain induced by IPTG was collected and disrupted by sonication. The crude protein extract was centrifuged at $13,000g$ at 4°C for 15 min. The supernatant was recovered and incubated with pretreated Ni-NTA beads for 1 h to extract the target protein by centrifugation at $700g$ at 4°C . The Ni-NTA beads adhering to the target protein were washed with a wash buffer thrice, and the target protein was eluted with an elution buffer and analyzed using gel electrophoresis.

2.4. Establishment of sensitization model of SD rats

Six SD rats were equally divided into two groups and respectively injected with PBS and allergy protein. Two milligrams of the rHum j1 protein in Freund's complete adjuvant was intraperitoneally injected into each rat. The inoculation was reinforced at the 2nd and 3rd weeks, while the control groups received PBS in Freund's incomplete adjuvant, according to the procedure described by Groneberg (Groneberg et al., 2003). The sera were placed at 20°C for 4 min and bathed in 37°C water for 15 min. After centrifugation at 3000 rpm for 15 min, the supernatant was salted out, and pellets containing IgG and IgE were obtained. The pellets were dissolved in PBS and an equal volume of 100% autoclaved glycerol was added. The solution was then stored at -20°C .

2.5. Western blotting

After the SDS-PAGE electrophoresis, the proteins were transferred onto NC membranes by electrical transfer. BSA (1%) was then added for 1 h to seal the membrane. The rat sera (1:100 diluted) was added and incubated at 37°C for 1 h. The membrane was then washed thrice (5 min each) with TBST (tris-buffered saline with Tween-20) buffer (pH 7.4). HRP-labeled second antibodies (1:5000 diluted) (YESEN Biotechnology Co. Ltd) were then added and incubated at 37°C incubate for 1 h. The membranes were then again washed with TBST buffer (pH 7.4). Finally, the substrate (tetramethyl benzidine, TMB) was added and incubated at 37°C for 10–30 min for color development. The gray value of the protein bands was analyzed using the Image J software, and histograms were generated using the GraphPad Prism5 software.

2.6. Exposure treatment of protein sample

The rHum j1 protein was treated with NO₂ and O₃ in an exposure chamber we designed (see [Supplementary Material Fig. S2](#)). Mass level of the exposure gaseous pollutants was adopted from a published report ([Lang-Yona et al., 2016](#)) and was modified. The exposure chamber was a sealed glass container (15.0 L) ([Lu et al., 2014](#)). 1 g protein (2 ml, 500 mg/ml) was transferred into a septic fresh-keeping film and exposed to NO₂+O₃, NO₂, and O₃ respectively. Initially, the mixed gas (400 ppb NO₂ +750 ppb O₃) was imported into the exposure chamber and reacted with the protein sample at 25 °C, avoiding light. After 3 h of exposure, considering mass concentration of the gases in the chamber decreases with the reaction proceeding, another mixed gas (200 ppb NO₂ +375 ppb O₃) was added into the chamber to ensure the full effect of pollutants on protein as much as possible. After 6 h of exposure, the protein samples were recovered from the filter for analysis. The sole gas exposure experiment followed the same procedure.

2.7. Mass spectrometric analysis

After exposure to NO₂+O₃, the purified rHum j1 protein was dissolved in PBS (pH 7.2) at a concentration of 0.1 mg/ml. After exposure, the rHum j1 protein was purified using a Ni column and separated and recovered by SDS-PAGE electrophoresis. It was then stored at −70 °C. After decolorization, the protein was added to 40 µl trypsin buffer and enzymolyzed for 16–18 h at 37 °C. The product of enzymolysis was collected onto a Thermo scientific EASY column (75 µm × 100 mm × 3 µm; C18) for desalination and separation with capillary high performance liquid chromatography (HPLC). Formic acid and formic acid acetonitrile (both 0.1%) were used as A and B solutions. The HPLC products were analyzed using the Q Exactive mass spectrometer (Thermo Fisher, USA). The data was searched using Mascot2.2 for the corresponding database (R554.fasta), and the identified protein results were extracted.

2.8. Protein structure characteristic analysis

The rHum j1 protein samples (0.1 mg), after the different treatments, were analyzed using a UV-vis spectrophotometer (UV759CRT, China) with a scanning wavelength of 250–420 nm and a scanning rate of 100 nm/min. The protein samples (0.1 mg) were dissolved in PBS for CD measurement using the Jasco J-815 spectropolarimeter (Jasco, MA, Japan) under the far-UV region from 190 to 250 nm. The ellipticity was recorded at a scanning speed of 500 nm/min, an accumulation of 3, and a bandwidth of 0.2 ± 0.1 nm.

2.9. Enzyme-linked immunosorbent assay

Microtiter plates (96-well) were coated with 200 µl of the protein sample and incubated for 20 h at 4 °C avoiding light (AL). After rinsing with PBS thrice, 100 µl of rat-derived antibodies (1:1000 dilution) was added into each well and incubated at 37 °C for 1 h AL. The plates were washed thrice, and 100 µl of horseradish peroxidase (HRP)-labeled second antibodies (1:5000 dilution) was added. The samples were again incubated at 37 °C for 0.5–1 h AL. After washing with PBS, 100 µl of freshly concocted TMB substrate solution was added to each well and incubated at 37 °C for 15 min AL. Finally, 50 µl of 2 M sulfate was added to terminate the reaction. The positive cutoff was considered 2.1-fold the optical density (OD; at 450 nm) of the negative control. SARS software was used for the statistical analysis.

2.10. Allergenicity in Balb/c mice

Twelve pathogen-free male balb/c mice (4 weeks of age) were equally divided into three groups. Each group of animals was respectively injected with PBS, unexposed protein and exposed protein. The operating procedure was as follows, the rHum j1 protein (0.2 mg) and isopyknic Freund's complete adjuvant were intraperitoneally injected into pathogen-free male Balb/c mice. One week later, the mice were injected with 0.2 mg of rHum j1 and isopyknic Freund's incomplete adjuvant. This procedure was repeated once a week. Mice in the control groups were administered PBS in Freund's incomplete adjuvant. Finally, the mice received a challenge dose of atomization treatment with pollen extracts every day for a week (30 min each).

Serum rHum j1-specific IgE and IgG, histamine, and cytokines were obtained and analyzed using a test kit (Nanjing Jiancheng Bioengineering Institute, China) and an ELISA kit (Westang Biotechnology Co., Ltd), according to the manufacturers' instructions.

2.11. Statistical analysis

Data were obtained from at least three independent experiments and expressed as mean ± standard error (SE). Statistical analysis for the paired Student's *t*-test and ANOVA were performed with SPSS version 11.5. Differences were considered statistically significant when *p* < 0.05.

3. Results

3.1. Prokaryotic expression and affinity chromatography for purifying rHum j1

The CDS sequence of the rHum j1 gene was cloned into pET-30a and the resultant prokaryotic protein expression construct was transformed into the *E. coli* strain, Rosetta. After IPTG-induced cultivation at 30 °C for 6 h to express the recombinant tandem six-histidine peptide (His₆)-fused Hum j1 (rHum j1), the bacterial culture was centrifuged. The pellet was the recovered, from which the protein was extracted by ultrasonication and purified using His6-tag affinity chromatography. The obtained protein samples were analyzed by SDS-PAGE gel electrophoresis. We found that rHum j1 mainly existed in the supernatant of the centrifuged cell lysate and had a molecular weight (MW) of 33 KDa, which was consistent with the predictions. Moreover, the rHum j1 band eluted from the affinity column was single and sharp ([Fig. 1A](#)), suggesting that the prokaryotic expression and tag affinity chromatography systems were successful for the enrichment and purification, respectively.

3.2. IgG and IgE recovered from rHum j1-immunized rats were suitable for specific detection of Hum j1

To obtain the antibodies against rHum j1, SD rats were inoculated with the purified rHum j1 solution (2 mg/ml) four times, after which aerosol pulverization was performed on the mixture of rHum j1 solution and *Humulus* pollen protein extract (PPE). Twenty-one days after pulverization, the rat sera were isolated from peripheral blood for analyzing IgG and IgE using HRP-conjugated goat anti-rat IgG antibody and murine anti-rat IgE antibody, respectively. The results of ELISA showed that the injection of rHum j1 resulted in SD rats producing the corresponding IgG and IgE antibodies; the combination of aerosol pulverization and injection resulted in a higher level of IgE production ([Fig. 1B and C](#)). Western blot analysis on the PPE revealed

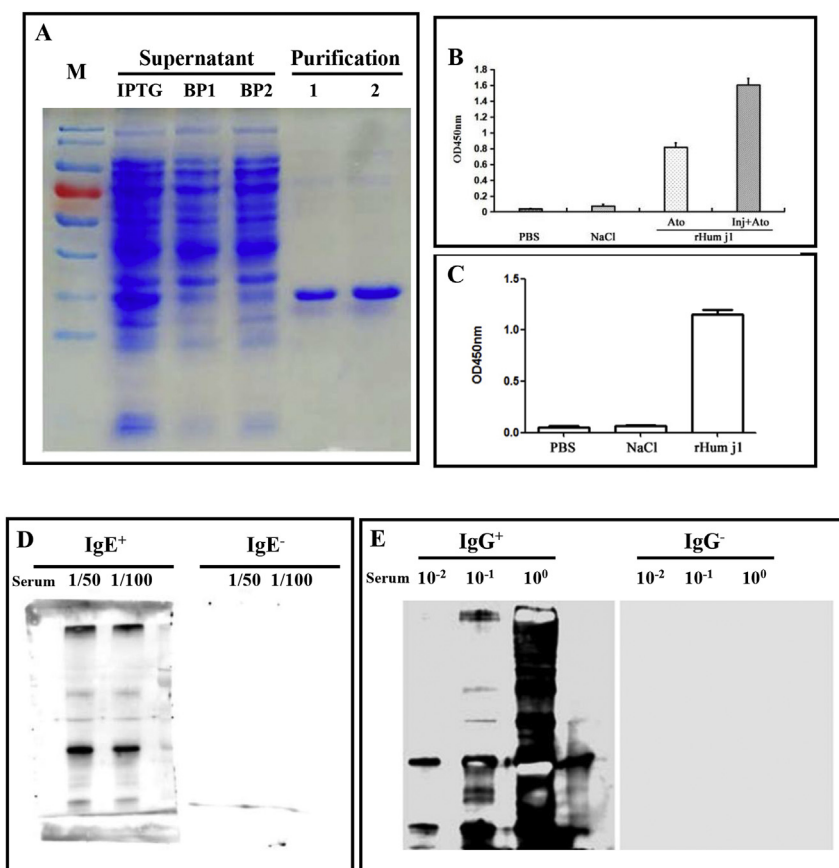


Fig. 1. Purification and antibody preparation of rHum j1.

(A) Purification of rHum j1 protein. (B). ELISA assay of IgE antibody against rHum j1 derived from rats. (C). ELISA assay of IgG antibody against rHum j1 derived from rats. (D). Western blot detection specificity of rat IgE antibodies to rHum j1 protein in *Humulus* pollen extract. Standard deviations are shown ($n > 3$, $\pm$ SD, $p < 0.05$ Student's *t*-test). All of the experiments presented here were performed at least 3 times, and similar results were obtained. BP1 and BP2= NO 1 and 2 sample pulled by beads; 1, 2 = the protein purified with BP1 and 2. PBS= PBS buffer, Ato = atomization, Inj = injection.

that natural Hum j1 protein bands (with MW of approximately 18 kDa) could be detected using both IgG and IgE antibodies of the immunized rats (Fig 1 D to E). With the continuous dilution of the serum, the impure protein bands could hardly be detected, and the Hum j1 band became predominant (Fig. 1D and E). Hum j1 is a profiling protein that is commonly found in all eukaryotic organisms. Therefore, in order to verify the specificity of the immunized rat IgG and IgE towards Hum j1, western blot assays were performed on PPEs from both *Humulus* and *Arabidopsis*. The results showed that the positive Hum j1 protein band was detected only in the *Humulus* PPE, and not in the *Arabidopsis seedling*, using the immunized rat IgG (Fig. 2A and B). Similarly, ELISA tests showed positive results only for *Humulus* samples, regardless of IgG or IgE antibodies in the rat sera (Fig. 2C and D). Further, the ELISA assay indicated that trace amounts of rHum j1 could be quantified using the IgG antibody from the immunized rats against rHum j1 (Fig. 2 E), and the minimum detection limit for the IgG antibody was 100–150 pg/ml (Fig. 2E). Based on the ratio of the MWs of rHum j1 and the natural Hum j1 (33:18), the detection limit for the natural Hum j1 using our prepared rat sera would be 55–85 pg/ml.

3.3. Allergenicity of rHum j1 increased after exposure to O₃/NO₂

There have been reports that joint exposure to NO₂ and O₃, which often occurs naturally in urban areas, could change the allergenicity of pollen allergens (Lang-Yona et al., 2016; Ribeiro

et al., 2017). To investigate this change, the rHum j1 was exposed to joint NO₂+O₃, as well as to sole NO₂ and O₃, using the protocol described by Lang-Yona et al. (2016). It was found that the joint NO₂+O₃ and sole O₃ exposure caused a significant increase in the binding capacity of rHum j1 to the immunized rat IgE, although the increase in binding ability to the immunized rat IgG was not significant (Fig. 3A and B). Compared to joint NO₂+O₃, sole NO₂ exposure seemed to hardly affect the IgG and IgE-binding ability of rHum j1 (Fig. 3 C). To investigate the change in allergenicity of rHum j1 exposed to joint NO₂+O₃, compared to non-exposed controls, Balb/c mice were immunized with the exposed and non-exposed rHum j1. The exposed rHum j1 were found to cause more severe allergic symptoms in mice. The level of IgE antibodies in the peripheral blood of mice immunized with the exposed rHum j1 was higher than that in the peripheral blood of mice immunized with the non-exposed rHum j1 (30% increase in mouse sera) (Fig. 3 D). In contrast, the IgG levels were lower in mice immunized with the exposed rHum j1 than in mice immunized with the non-exposed rHum j1 (Fig. 3 D). In addition, the levels of TNF- α and IL-4 (Th2 type cytokines) in mice immunized with the exposed rHum j1 were higher than those in mice immunized with the non-exposed rHum j1 (Fig. 3 E). However, the levels of histamine released and IFN- γ (Th1 type cytokines) were similar in the mice immunized with exposed and non-exposed rHum j1 (Fig. 3 F). Taken together, these observations indicated that the allergenicity of the exposed rHum j1 was significantly higher than that of the non-exposed rHum j1.

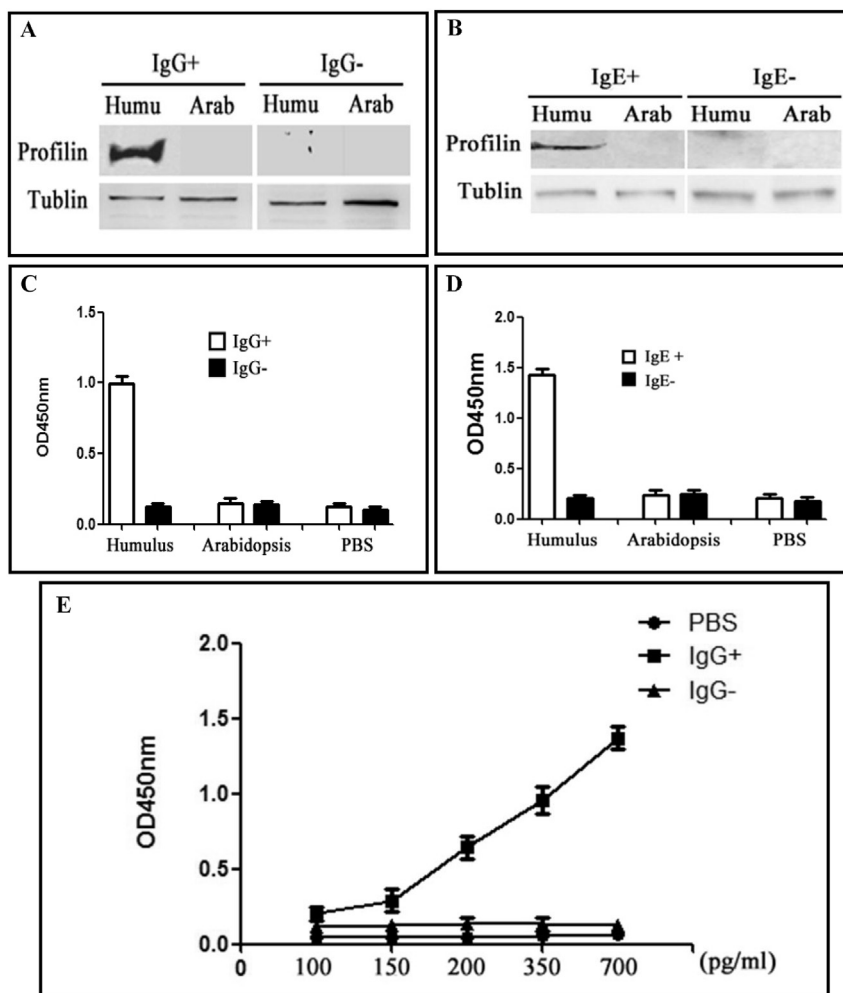


Fig. 2. Detection of anti-rHum j1 antibody titer and specificity.

(A) The rat IgG antibodies used to detect the Hum j1 in protein samples from different sources. (B) The rat IgE antibodies used to detect the Hum j1 in protein samples from different sources. (C) ELISA analysis of specificity of rat IgG antibody to Hum j1. (D) ELISA analysis of specificity of rat IgE antibody to Hum j1. (E) ELISA analysis of rat IgG antibody titer. Standard deviations are shown ($n > 3$, $\pm$ SD, $p < 0.05$ Student's t -test). All of the experiments presented here were performed at least 3 times, and similar results were obtained.

3.4. Joint $\text{NO}_2 + \text{O}_3$ exposure changed the structure of rHum j1 through novel disulfide bond formation

In order to investigate the effect of joint $\text{NO}_2 + \text{O}_3$ exposure on the structure of rHum j1, a tandem LC-MS/LC spectrometric assay was performed. The results showed that the exposure oxidized sulfhydryl groups of the neighboring Cys¹⁴⁰ and Cys¹⁴¹ to form a novel disulfide bond between them (Table 1, Fig. 4 A, B); unexpectedly, no nitration by NO_2 was detected. Disulfide bonds play an important role in stabilizing advanced protein structure, and disruption of disulfide bonds can decrease protein homeostasis (Vinther et al., 2013; Karimi et al., 2016; Kwon et al., 2017). To establish the consequences of such novel disulfide bond formation on the protein structure, further ultraviolet (UV) and circular dichroism (CD) spectroscopy assays were performed. The results showed that the UV absorption value of the exposed rHum j1 was significantly higher than that of unexposed control (Fig. 4 C), suggesting that the overall protein structure of the exposed rHum j1 was looser than that of the non-exposed one. Furthermore, the CD assay on the secondary structure demonstrated that the number of alpha helixes was decreased, while that of the parallel/anti-parallel beta-strands and random coils was increased by joint O_3/NO_2 exposure (Fig. 4D and E). In addition, many of the non-exposed

rHum j1 proteins formed pellets after repeated freezing-thawing, and its UV absorption value at 280 nm dropped more dramatically than that of the exposed rHum j1, suggesting that protein homeostasis was reinforced by joint O_3/NO_2 exposure (Fig. 4 F).

4. Discussion

Hum j1 is a dominant allergen in the *Humulus* pollen. Effective enrichment and purification of the Hum j1 protein, as well as the production of its antibodies, are important for investigating the mechanism of *Humulus* pollen allergy, to prevent pollen sensitization, and establish an effective monitoring system. The IgG and IgE obtained from the rHum j1-immunized rats had high titers and high specificity. Subsequent experimental analyses showed that these rat-derived antibodies could be used to detect trace amounts of Hum j1 proteins, with concentrations as low as 110–170 pg/ml. The allergenic protein content in the air during the pollen season frequently reached 1–20 pg/m³ (Buters et al., 2015). Therefore, the antibodies produced in this study would be suitable for efficiently detecting trace amounts Hum j1 on aerial particulate matter.

We also found that $\text{NO}_2 + \text{O}_3$ treatment could cause structural changes in Hum j1 by oxidizing the sulfhydryl group of Cys¹⁴⁰ and Cys¹⁴¹ to form a disulfide bond, which increased the affinity to IgE.

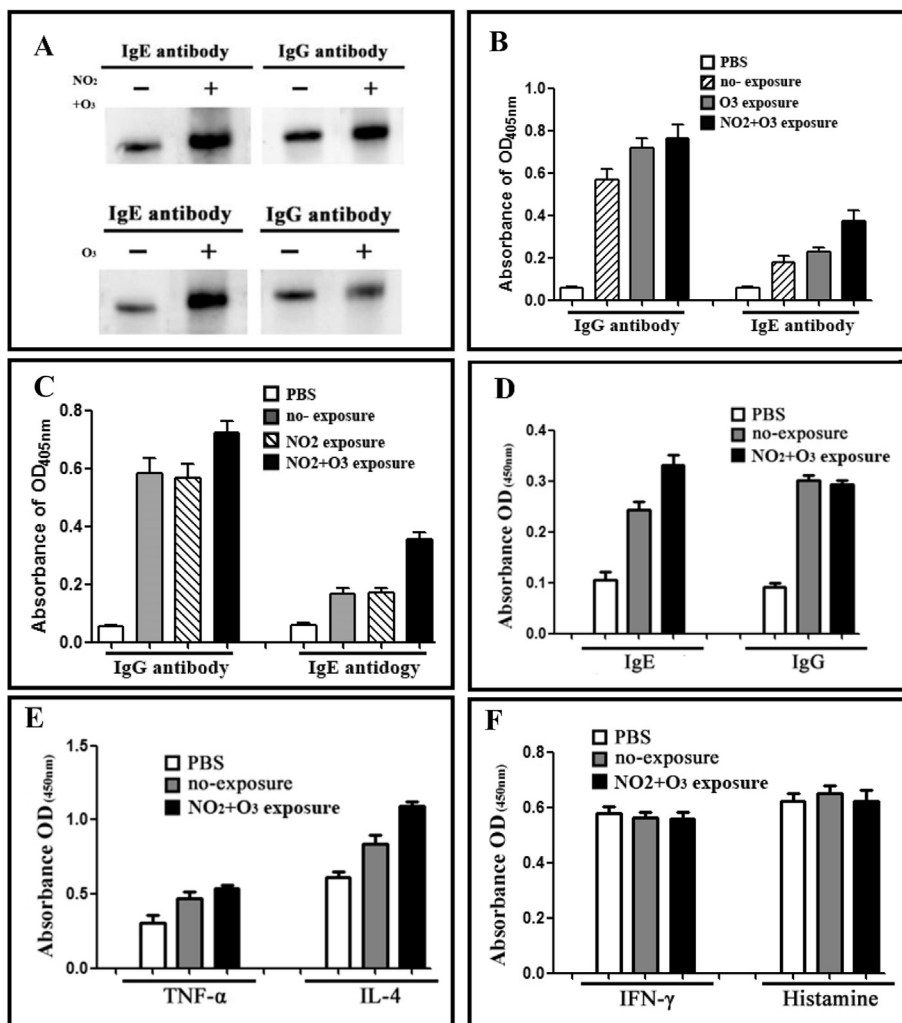


Fig. 3. Effect of NO_2+O_3 exposure on the Allergenicity of rHum j1 protein.

(A) Western blot analysis the immune binding ability of rHum j1 to antibodies. (B) ELISA assay of the immune binding ability of IgG and IgE antibody to rHum j1 with O_3 and NO_2+O_3 exposure. (C) ELISA assay of the immune binding ability of IgG and IgE antibody to rHum j1 with NO_2 and NO_2+O_3 exposure. (D) Levels of IgE and IgG antibodies in serum of Balb/c mice. (E) Levels of $\text{TNF-}\alpha$ and IL-4 in serum of Balb/c mice. (F) Levels of IFN- γ and Histamine in serum of Balb/c mice. Data are expressed as mean SD (n = 3). The Y-axis is the ultraviolet absorption value of 450 nm.

Table 1
Results of protein mass spectrometry.

Order	Sequence	Spectrum	Score	Calc_M	Delta	ppm	modification	Sample ID	Engine	Rank	Proteins
Protein without NO_2/O_3 exposure											
Protein with NO_2/O_3 exposure											
1 ^a	CCGQPLGFDR(1,2)										
1,1	R554-CHU.10474.10474.2.dta		2.93E-25	1173.49389	-0.00037	-0.315298	1,C(C-1);2,C(C-1)	1.pFind	1.pFind	1	R554(140,141)
2,1	R554-CHU.10783.10783.2.dta		5.66E-25	1173.49389	-0.00139	-1.184497	1,C(C-1);2,C(C-1)	1.pFind	1.pFind	1	R554(140,141)
3,1	R554-CHU.10705.10705.3.dta		5.76E-22	1173.49389	-0.00026	-0.221561	1,C(C-1);2,C(C-1)	1.pFind	1.pFind	1	R544(140,141)
4,1	R554-CHU.11017.11017.3.dta		6.14E-15	1173.49389	-0.00158	-1.346407	1,C(C-1);2,C(C-1)	1.pFind	1.pFind	1	R544(140,141)
5,1	R554-CHU.10385.10385.3.dta		5.65E-12	1173.49389	-0.00326	-3.084805	1,C(C-1);2,C(C-1)	1.pFind	1.pFind	1	R544(140,141)

^a Means two sulphhydryl groups were oxidized to form a disulfide bond.

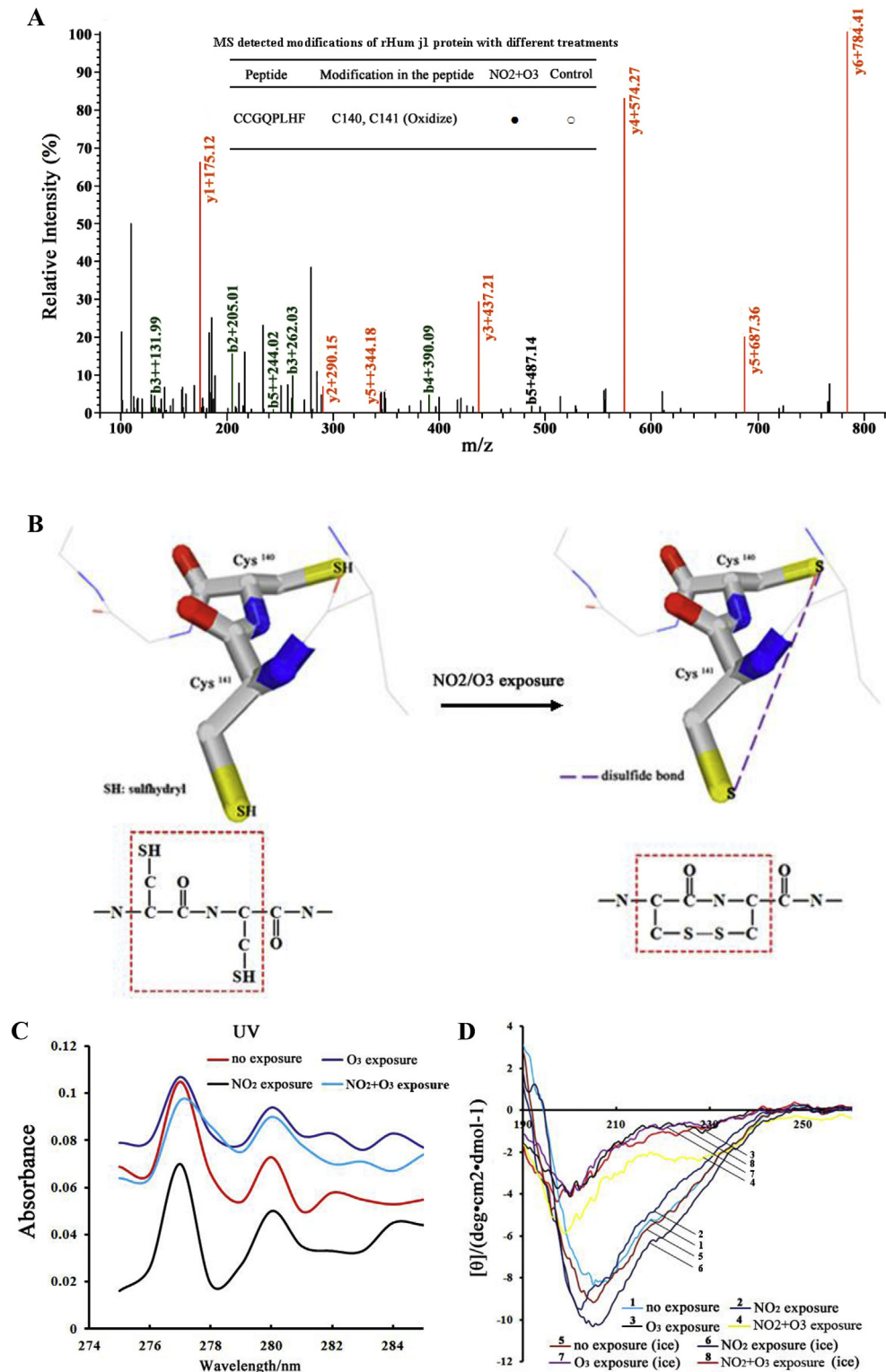


Fig. 4. Effect of NO₂/O₃ exposure on the structure of Profilin protein.

(A) A typical mass spectrum to identify oxidation of rHum j1 protein; (B) A sketch map of the formation of disulfide bond between Cys¹⁴⁰ and Cys¹⁴¹ in rHum j1 by NO₂/O₃ exposure; (C) Absorbance values at 280 nm of rHum j1 Profilin with different treatment; (D) CD spectra of rHum j1 Profilin with different treatment; (E) The alteration of conformational units after exposure processing; (F) Effects of NO₂/O₃ exposure on the stability of Profilin protein. Data are expressed as mean SD (n = 5). **Peptide**: The sequence with modifications which detected by MS; **Modifications in the peptide**: all the modifications in this peptide; **NO₂/O₃**: protein with NO₂/O₃ exposure, **Control**: protein without NO₂/O₃ exposure, results of 3 independent experimental tests; ●: modifications detected in this peptide; ○: modifications not detected in this peptide; Oxidize means the formation of the disulfide bond.

Furthermore, when treated by repeated freezing and thawing, the exposed protein showed better homeostasis than the non-exposed one, as evidenced by the formation of lesser denaturated protein pellets. In addition, it was further verified that the overall immune responses of the exposed rHum j1-immunized Balb/c mice were more significant than the non-exposed rHum j1-immunized ones. Taken together, these observations confirmed that exposure to joint O_3+NO_2 augmented the allergenicity of rHum j1.

Besides NO_2+O_3 , rHum j1 was also exposed to sole O_3 and NO_2 . The effect of sole O_3 exposure was similar to that of joint NO_2+O_3 , as evidenced by the similar curve patterns of the UV spectrum and CD spectroscopy (Fig. 4C and D). However, the sole NO_2 exposure seemed to affect the rHum j1 conformation more than the O_3 -involved exposures. In contrast to the looser overall conformation caused by joint O_3/NO_2 or sole O_3 exposure, NO_2 exposure made the overall rHum j1 conformation more compact than the non-exposed rHum j1. Consistent with this trend in structural changes, the allergenicity of rHum j1 seemed to decrease after NO_2 exposure. Thus, it is reasonable to deduce that it is the O_3 exposure that primarily causes the sulfide bond formation, and that the effects of NO_2 exposure were probably overridden by O_3 .

Although a His₆ tag was added at the -N end of Hum j1 during the expression of rHum j1, protein structure studies confirmed that Cys¹⁴⁰ and Cys¹⁴¹ near the -C end were affected, resulting in the changes in the structure and properties of rHum j1. Based on previous protein studies (Vahedi et al., 2011; Pichler et al., 2014; Liu et al., 2015) and the fact that rHum j1 retained the immunogenicity and allergenicity of Hum j1, the effect of the additional tags should be negligible. In the secondary structure of rHum j1, the novel disulfide bond pulls the side chains of two cysteine residues closer, forming an internal ring comprising 8 atoms along the main chain of the protein (Fig. 4 A). This internal ring disrupts the continuity of the alpha helix, and leads to the consequences demonstrated in the result of the CD assay, i.e., decreases in the number of alpha-helices in the exposed rHum j1. Consequently, the proportion of beta-strands and random coils increased, resulting in the exposed rHum j1 having a looser overall structure than the non-exposed one. However, the formation of the novel internal ring, a stable structure, might increase the overall area of the IgE-interacting sites, resulting in increased binding affinity to IgE. Alternatively, it is known that novel sulfide bond formation makes the overall rHum j1 structure looser. It is possible that the looser overall rHum j1 structure facilitates the exposure of more internal epitopes, thus increasing the allergenicity.

Previous studies have reported novel disulfide bonds between two molecules of a profilin from the rubber tree, rHev b8 (Mares-Mejia et al., 2016). They found that higher concentrations of rHev b8 protein solution (10 mg/ml) directly promoted the formation of a novel disulfide bond between two monomeric rHum j1 subunits of the dimeric form, which could be detected by non-reductive SDS electrophoresis. The amino acid sequence alignment assay between Hum j1 and Hev b8 showed that these two proteins shared a low level of homology (Fig. S3). Therefore, the conformation of rHum j1 cannot be predicted based on the resolved conformation of rHev b8. Furthermore, the cysteine residues, whose sulfhydryl groups form the disulfide bond in rHev b8, are absent in the rHum j1. However, it is still attractive to consider the possibility that joint NO_2+O_3 exposure could accelerate homodimer formation of many plant rHum j1 proteins, as most of them share the conserved cysteine residues of rHev b8, whose sulfhydryl groups can be oxidized to form disulfide bonds between monomeric rHum j1 molecules. As for Hum j1, intra-molecular, rather than inter-molecular, disulfide bond is likely formed in the rHum j1, probably due to the spatial proximity of the sulfhydryl groups of neighboring cysteine residues, although novel sulfide bond formation leads to increased

allergenicity in both cases.

Air pollution aggravating pollen allergy disease has been widely recognized (Pazmandi et al., 2012; Ackaert et al., 2014; Kampf et al., 2015; Silva et al., 2015; Ribeiro et al., 2017; Zhao et al., 2017). Air pollutants could make pollen easier to break down and release more allergens, the immunogenicity and sensitization of allergen proteins could be enhanced to varying degrees. For example, Kampf et al. reported that cross-linking allergen proteins could be induced by pollutants, and resulting in high protein structure changes, thus affected the allergenicity (Kampf et al., 2015). However, our study demonstrated that NO_2+O_3 exposure did not cause cross-linking between Profilin protein molecules, and did not result in the formation of large molecular weight protein bands (Fig. S4). The formation of a disulfide bond within a single protein molecule, resulting in changes on the allergenicity and immunogenicity of the protein.

5. Conclusions

In conclusion, joint NO_2+O_3 exposure, the main form of air pollution in widespread areas of the most populated cities in the world, can lead to the formation of a novel disulfide bond, which not only stabilizes the structure of allergens, enabling them to remain in the air for a longer time, but also enhances their allergenicity. Our findings could provide new clues for elucidating the biochemical mechanism behind air pollution-induced increase in pollinosis prevalence.

Competing financial interest declaration

The authors declare they have no actual or potential competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envpol.2017.11.078>.

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Supplemental figures

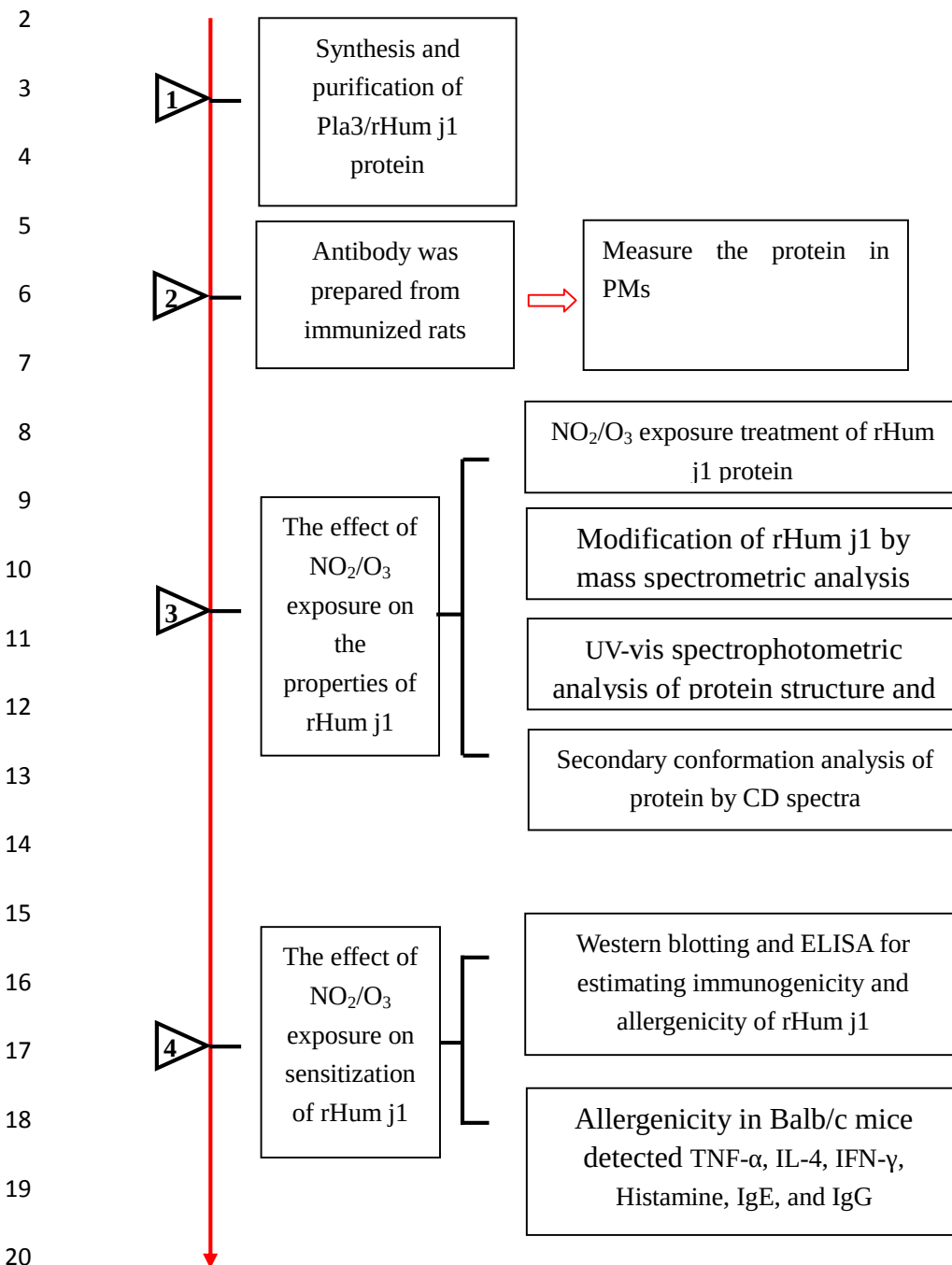


Figure 1S. Schematic diagram of this study

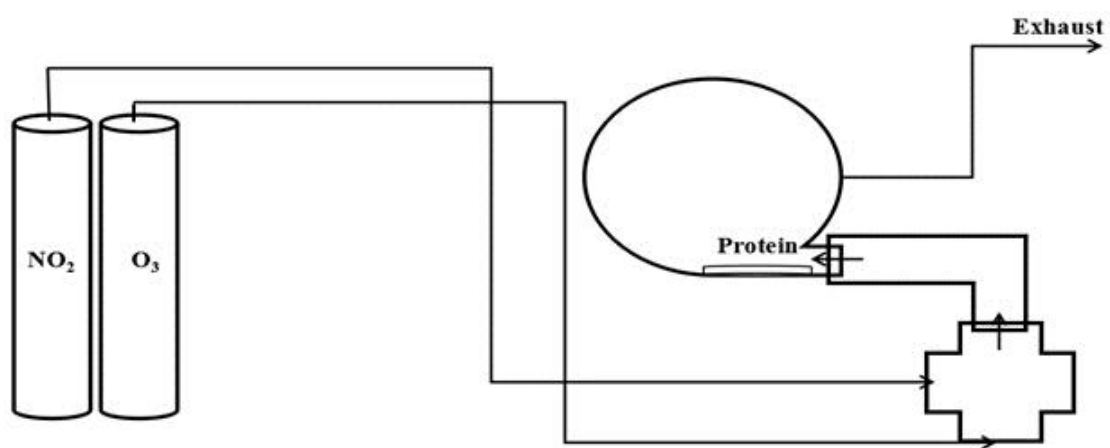


Figure S2. Schematic diagram of the exposure chamber.

A

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pdb|rHev b 8|B  - - - - - MSWQAYVDD - - - HLMCEIEGNHLSAAAIIIG 27
pdb|rZea m 12|A  - - - - - MSWQAYVDD - - - HLLCDIEGQHL SAAAIVG 27

pdb|rHev b 8|B  QDGSVWAQSANFPQFKSEETGIMSDFHEPGTLAPTGLYI 67
pdb|rZea m 12|A  HDGSVWAQSENFPPELKPEEVAGMIKDFDEPGTLAPTGLFV 67

pdb|rHev b 8|B  GGTKYMVIQG - - EPGAVIRGKKGP GGVTVKKTNQALIIG - 104
pdb|rZea m 12|A  GGTKYMVIQG - - EPGVIRGKKGT GGITIKKTGMSLIIG - 104

pdb|rHev b 8|B  - - - IYDEPMT PGQCNMIVERLGDY LIDQGY - - - - 131
pdb|rZea m 12|A  - - - IYDEPMT PGQCNMVVERLGDY LIEQGF - - - - 131

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B

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pdb|rHev b 8|B  - - - - - MSWQAYVDD - - - HLMCEIEGNHLSAAAIIIG 27
pdb|rZea m 12|A  - - - - - MSWQAYVDD - - - HLLCDIEGQHL SAAAIVG 27
pdb|rHum j1     MKNLQHHNK I I T F K T P D D S T T L E L N S H I S L P Y H W E Q C I D L 40

pdb|rHev b 8|B  QDGSVWAQSANFPQFKSEETGIMSDFHEPGTLAPTGLYI 67
pdb|rZea m 12|A  HDGSVWAQSENFPPELKPEEVAGMIKDFDEPGTLAPTGLFV 67
pdb|rHum j1     KTGEIYYINWRNGMKAKEDPRTTITTEYNNSNGSNGDYYYSE 80

pdb|rHev b 8|B  GGTKYMVIQG - - EPGAVIRGKKGP GGVTVKKTNQALIIG - 104
pdb|rZea m 12|A  GGTKYMVIQG - - EPGVIRGKKGT GGITIKKTGMSLIIG - 104
pdb|rHum j1     EDSSYDSEESSSESSPPPATTSRESQYRGAEKDHVLLVVG 120

pdb|rHev b 8|B  - - - IYDEPMT PGQCNMIVERLGDY LIDQGY - - - - 131
pdb|rZea m 12|A  - - - IYDEPMT PGQCNMVVERLGDY LIEQGF - - - - 131
pdb|rHum j1     CKSCFMYFMV PKQVQECPKCCGQPLHFDRSQSGSQ 155

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Figure S3. Homology analysis of rHum j1

(A) Homologous comparison analysis of rHev b8 and rZea m12. (B) Homologous comparison analysis of rHev b8, rZea m12, and rHum j1.

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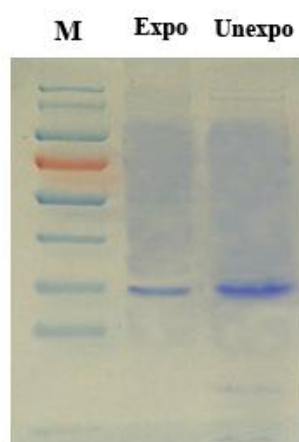


Figure Supplied 4. Native-PAGE electrophoresis analysis of the molecular weight of profilin protein after NO₂+O₃ exposure.

M: marker; Expo: NO₂+O₃ exposure; Unexpo: without exposure treatment