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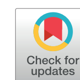
Allergenicity of recombinant *Humulus japonicus* pollen allergen 1 after combined exposure to ozone and nitrogen dioxide☆



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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Ozone (O_3) and nitrogen dioxide (NO_2) are thought to play primary roles in aggravating air pollution-induced health problems. However, the effects of joint O_3/NO_2 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 O_3/NO_2 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 O_3/NO_2, 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 O_3/NO_2, 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 O_3, 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; Platts-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.

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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; Gruijthuijsen et al., 2006; Ackaert et al., 2014; Kampf et al., 2015). Ragweed pollen allergen, Amb, showed differential transcript expression when exposed to NO_2+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 NO_2+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-μm 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 (Sgma-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′-TGCCATGGCTAAGAACT TGCAGCATCACAACA-3′ and 5′-CCAAGCTTGTTGAGAGCCACTTTGA-GATCGATC-3′. It was then double-digested with *Nco*I and *Hin*dIII, 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 13,000*g* 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 700*g* 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.

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