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Studies on relationships between air pollutants and allergenicity of *Humulus Scandens* pollen collected from different areas of Shanghai

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ABSTRACT

Pollen pollution and allergy are becoming prominent issues in China. However, few studies on pollinosis have been reported. As an allergen in the atmosphere, allergenic *Humulus scandens* pollen was collected from four districts of Shanghai, including Wusong (WS), Jiading (JD), Xujiahui (XJH) and Songjiang (SJ). The mass concentrations of SO_2, NO_2, O_3, $PM_{10,}$ and $PM_{2.5}$ (particulate matter with air dynamic diameter less than 10 and 2.5 µm, respectively) near the four sampling sites were also recorded during *Humulus scandens* pollen season. The allergenicity of the *Humulus scandens* pollen was assessed by using of a rat model and enzyme linked immunosorbent assay (ELISA). Relationships between the allergenicity and air pollutants were correlated. Our results demonstrated that the biological viability of the pollens collected from the four districts exhibited no significant differences. ELISA and dot blotting results further demonstrated that the serum of sensitized rats exhibited much higher immune-reactive response than that of control groups. Western blotting showed that the 15 KD (1KD = 1000 dalton) proteins of *Humulus* pollen led to the allergic response. The allergenic intensity of *Humulus* pollen protein from different samples followed the pattern: WS > JD > XJ > SJ. There was a negative relationship between the allergenicity of *Humulus* pollens and PM_{10} ($R = -0.99$) / $PM_{2.5}$ ($R = -0.73$), and a positive relationship with O_3 ($R = 0.92$). These data clearly showed that PM_{10} and $PM_{2.5}$ could enhance *Humulus* pollen protein release, and O_3 could aggravate the allergenicity of the *Humulus* pollen.

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Introduction

Pollinosis is an increasingly widespread allergic disease, which is triggered by many kinds of pollen grains (Ghosh et al., 2015; Zhou et al., 2019). More and more people are suffering from respiratory allergy diseases caused by pollen grains in the outdoor environment (Buter et al., 2015). The incidence of allergic disease is 30%–40% worldwide, and has doubled in the last 30 years (Traidl-Hoffmann et al., 2003), particularly in industrialized countries. The immune response in the human body is a defense system which protects an individual from foreign pathogen invasion. However, in some cases, the immune system will exhibit hypersensitivity to certain non-pathogenic substances such as plant pollens, and an allergic reaction will occur (Uzzaman and Cho., 2012; Escribese et al., 2015). According to the mechanism and incidence, the allergic reactions are always classified into four types, i.e. IgE (Immunoglobulin E)-mediated hypersensitivity, antibody-dependent cytotoxic hypersensitivity, immune complex-mediated hypersensitivity and delayed T cell (Thymusdependent lymphocytes cell)-mediated hypersensitivity (Uzzaman and Cho., 2012; Yamada et al., 2014; Khorooshi et al., 2015; Petrisor et al., 2015). The type I hypersensitive reaction is based on the action of immunoglobulin E (IgE) antibodies against allergens (Traidl-Hoffmann et al., 2009; Rouvinen et al., 2010).

Many anemophily grass pollens are common sources of allergens worldwide. As a perennial herb, *Humulus* is a typical anemophily grass that is widespread in most areas of China, with a flowering period from August to October each year (Park et al. 2001). Since *Humulus japonicus* pollen in the air was first identified as an allergen source in 1965, more *Humulus* species pollens have been found to be allergens. For example, *Humulus scandens* is considered to be a principle allergenic plant, with its pollen widespread during autumn in Asian countries (Jeong et al., 2013; Jin et al., 2013). A recent survey of allergic diseases showed that pollen allergy is associated with the type I hypersensitive reaction, such as intermittent rhinitis (Li et al., 2009, 2012). Although there are many reports on the identification and *in vitro* expression of the allergen proteins of *Humulus scandens*, it is not clear whether the allergenicity of *Humulus* pollen is affected by air pollutants, such as NO_2, SO_2, O_3 and particulate matters. Therefore, this study aimed to explore the variation of allergenicity of *Humulus* pollen as affected by air pollutants in different areas of Shanghai, and tried to provide fundamental data for elucidation of the mechanism of pollinosis induced by the ambient allergens.

1. Materials and methods

1.1. *Humulus pollen collection and viability observation of the pollens*

Mature inflorescences with opening flowers of *Humulus* were collected from four districts of Shanghai: Wusong (E121°47′66″; N31°38′05.67″), Jiading (E121°40′58.55″, N31°32′01.8″ 2), Xujiahui (E121°43′47.22″, N31°17′86.01″) and Songjiang (E121°21′76.71″, N31°67′84″) (Fig. 1). The inflorescence pollens were dried at 30°C for 24 hr, and the pollen grains were collected from the anther. The pollen grains were filtered by using a mesh with pore size 40 µm, and then were kept in Eppendorf tubes at -20°C until use. The viability of pollens was tested using the Alexander staining assay according to Zhang et al. (2006), i.e., pollen grains were immersed in 50 µL staining solution in an Eppendorf tube for 6 hr at 40°C, and washed with 10% ethyl alcohol 2 times; about 5 µL pollen solution was transferred onto to a glass slide. Then the number of fertile pollen grains on each slide was obtained by light microscopy. The statistical data was derived from three independent experiments, and expressed as mean ± standard error (SE).

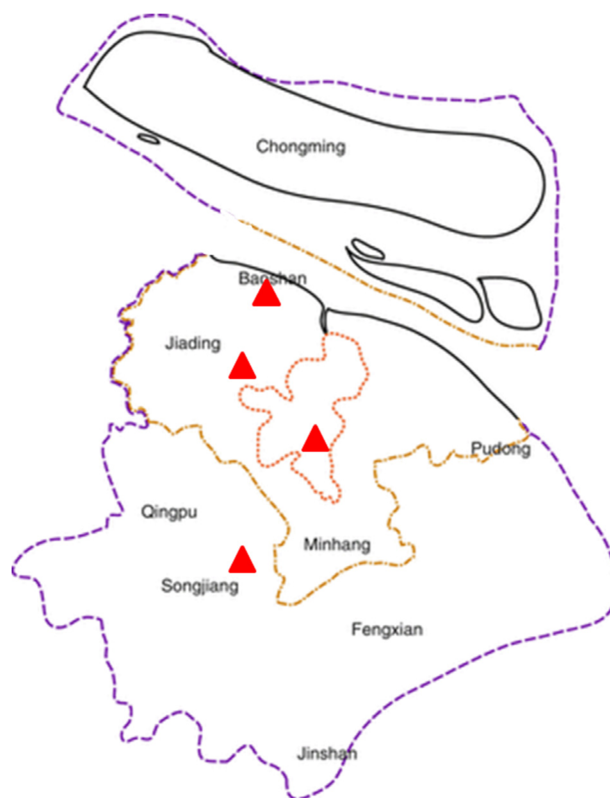

Fig. 1 – Sketch map of *Humulus* pollen sampling sites in Shanghai.

The mass concentrations of SO_2, NO_2, O_3 and PM_{10}, $PM_{2.5}$ in the atmosphere of the four districts during the *Humulus* pollen season (October 2014) were from the website of the Shanghai Environmental Monitoring Center, the data were used to analyze relationships between the allergenicity of *Humulus* pollen protein and the main air pollutants.

1.2. *Extraction and quantification of pollen protein*

Pollen (0.15 g) and 0.3 g carborundum were mixed together in a mortar and ground into powder with liquid nitrogen; 1 mL sodium borate buffer with 1 mmol/L phenylmethanesulfononyl fluoride (PMSF) was added into the mortar to homogenize the mixture, then the homogenized solution was transferred into a 2 mL Eppendorf tube and shaken (250 r/min) for 10 hr at 37°C. After the tube was centrifuged (4°C, 12,000 r/min) for 10 min, the supernatant was removed from the tube. The total protein was quantified by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher, USA).

1.3. *Establishment of pollen allergic Sprague–Dawley (SD) rat model*

Humulus pollen (5 g) was weighed in a beaker, and degreased by ethyl ether until the pollen became colorless. Coca's extract buffer (100 mL) was added and stirred for 60 hr at 4°C, and then centrifuged for 30 min at 4°C. The supernatant was transferred

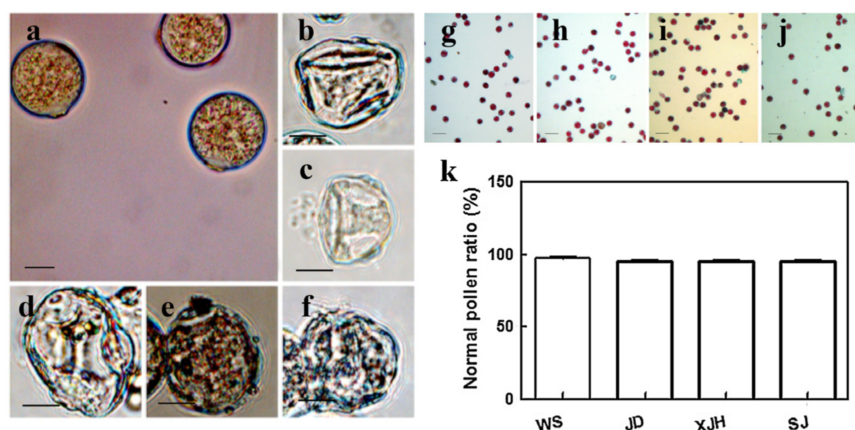

Fig. 2 – Characteristic of (a) the normal and (b–f) the abnormal *Humulus* pollen collected in Shanghai different areas (bar = 10 μm in (a)-(f)), Alexander stain of *Humulus* pollen collected from (g) Wusong (WS), (h) Jiading (JD), (i) Xujiahui (XJH) and (j) Songjiang (SJ), and (k) fertile pollen ratio (number (n) > 20, ± SD (standard deviation); p value (p) < 0.05).

into a dialysis bag. The dialysis bag was immersed in 0.7% normal saline (NS) for 48 hr at 4°C; NS was used 3 times, and then the dialysis product was placed in 2 mL Eppendorf tubes and stored at -20°C until used. Six Sprague-Dawley (SD) rats were purchased from Shanghai Sliker centre of Experimental Animals. They were divided into 3 groups: 2 rats were injected with pollen protein extract (PPE), 2 rats were treated with 0.7% normal saline (NS) and Freund's Adjuvant (control group), and another 2 rats were raised as usual (blank group). All the rats were housed under pathogen-free conditions within the animal care facility in the lab animal room of Shanghai University. All animal experiments were approved by the Animal Experiments Committee of the School of Life Science, Shanghai University. Reinforced inoculation was carried out on the 2nd and 3rd weeks, according to the procedure described by 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 r/min for 15 min, the supernatant was salted out, and pellets containing IgG (immunoglobulin G) were obtained. The pellets were dissolved in phosphate buffered solution (PBS) and an equal volume of autoclaved 100% glycerol was added. The solution was then stored at −20°C (Hong et al., 2018). Human serum containing IgE antibody (the patients were diagnosed as having pollinosis) was provided by Shanghai Xuhui Center Hospital.

1.4. *Enzyme linked immunosorbent assay quantification of allergenic intensity of Humulus pollen extract*

The pollen protein extract (PPE) from different samples was diluted by using embedded buffer until the final total protein concentration was 10 μg/mL. Diluted PPE (100 μL) was added into wells of polystyrene plates, and incubated for 20 hr at 4°C avoiding light (AL). Then the contents in each well were discarded and PBS buffer was added and incubated for 3 min, and all the supernatant in the wells was thoroughly removed. The wells were washed three times with PBS, and then 100 μL rat antibody (with 200-fold dilution) was added, incubated at 37°C for 1 hr AL, and washed three times with PBS. Horseradish peroxidase (HRP)-labeled second antibody (100 μL) (with a 3000–5000-fold dilution) was added, and incubated at 37°C for 0.5–1 hr AL, washing three times. freshly prepared tetramethyl benzidine (TMB) substrate solution (100 μL, 100 μg/mL) was added and incubated at 37°C AL for 10–30 min. Finally, 50 μL 2 mol/L sulfate was added to terminate the reaction, and the optical density (OD) of each well at 450 nm was recorded on a microplate reader (iMark, Petool, USA).

1.5. *Dot blot and western blot assays*

PPE (1–5 μL) was transferred onto a nitrocellulose (NC) membrane, and kept for 20 min at 37°C mins for the dot blot assay. The proteins were transferred onto the NC membrane by the method of electrical transfer after the sodium dodecyl sulfate polyacrylamide (SDS-PAGE) electrophoresis. Then, 1% BSA was added to seal the membrane for 1 hr; after that, 1:100 diluted rat sera was added and incubated at 37°C for 1 hr AL. The membrane was washed with PBS buffer 3 times. The 1:10,000 diluted HRP-labeled second antibody was added, and incubated at 37°C for 1 hr AL, and then the membrane was washed with PBST buffer (pH = 7.4). Finally, the substrate TMB (100 μg/mL) was added and incubated at 37°C for 10–30 min for the color reaction. The gray value of the protein bands was analyzed by Image J software (V1.8.0, National Institutes of Health, USA), and the data were analyzed using GraphPad Prism5 software (Version 5.0, GraphPad Software, USA).

2. Results

2.1. *Morphology of Humulus pollen*

The morphology of *Humulus* pollens is shown in Fig. 2. The normal *Humulus* pollen exhibited a nearly globular shape and light-yellow color with radius around 25 μm. The pollen grains showed a tough and smooth wall surface (Fig 2a). However, abnormal pollen grains, such as shrunken pollen (Fig. 2b), and broken grains (Fig. 2c, d, e, and f) could be found under light microscopy. It was noted that sub-pollen particles could be found to be released from the pollen (Fig. 2c). Alexander staining results revealed that the total viability of *Humulus* pollens from Wusong (WS) (97.32% ± 3.02%), Jiading (JD) (95.71% ± 1.92%), Xujiahui (XJH) (95.14% ± 2.37%) and Songjiang (SJ) (95.24% ± 3.77%) districts exhibited no significant difference (Fig. 2g-k).

2.2. *SDS-PAGE assay of Humulus pollen protein extract (PPE)*

The total amount of protein released from 1 mL *Humulus* pollen solution collected from XJH, JD, WS and SJ was 319.20 ± 32.90, 485.60 ± 33.89, 519.80 ± 28.46, 312.60 ± 27.40 μg, respectively (Fig. 3a). The WS and JD pollens released more protein than the XJH and SJ pollens.

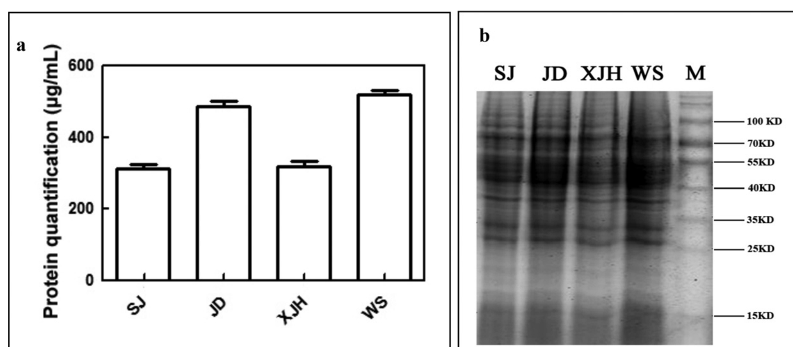

Fig. 3 – Analysis of *Humulus* pollen proteins in different regions of shanghai. (a) determination of pollen protein content in different regions; (b) Sodium dodecyl sulfate polyacrylamide-gel electrophoresis assay of protein extracted from *Humulus* pollen of different regions. M: marker; KD: 1000 dalton.

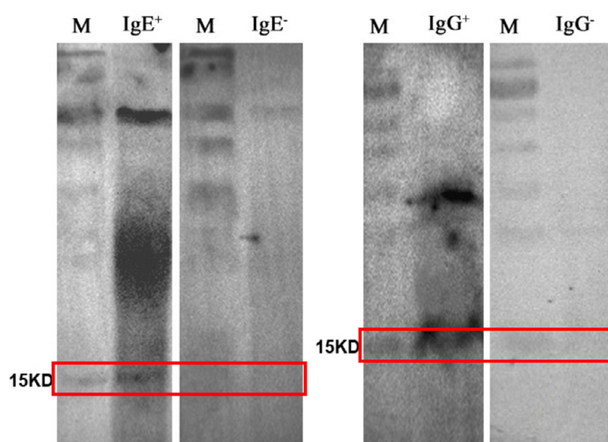

Fig. 4 – Western blot assay of sensitized protein in the *Humulus* pollens. The 15 KD protein bands of *Humulus* pollen showed distinct immune imprinting against human serum (IgE) and sensitized rat serum (IgG) compared to non-sensitized serum. IgE^+: serum containing IgE antibody against *Platanus acerifolia* allergen 3 (*Pla* a3); IgE^-: serum without IgE antibody against *Pla* a3; IgG^+: serum containing IgG antibody against *Pla* a3; IgG^-: serum without IgG antibody against *Pla* a3.

The same amount of protein was used for the SDS-PAGE assay. The molecular weights of 15, 27, 38, 70 and 100 KD (1 KD = 1000 dalton) were identified (Fig. 3b). Remarkably, the 100 KD protein band from XJH and WS samples exhibited more smearing than that of SD and JD samples, suggesting that the protein degradation in the WS and XJH samples was more serious.

2.3. *Allergic symptoms of sensitized SD rat model*

The allergic symptoms of sensitized SD rats appeared after 5 days of stimulus challenge with pollen protein extract (PPE) aerosol. The ear-scratching, hair-licking and sneezing frequency of the sensitized SD rats during the 5th-7th days (after exposure to PPE aerosol) showed that these allergic responses of the sensitized group were significantly more intensive than those of the control group and blank groups (Appendix A Fig. S1). Interestingly, a significant decline in allergic response could be observed on the 6th day.

2.4. *Western blot assay*

The western blot results demonstrated that the 15 KD band of *Humulus* pollen proteins showed specific immune-reactive responses against sensitized rat serum and human serum (Fig. 4), suggesting that the protein with molecular weight of 15 KD was allergenic.

2.5. *ELISA assay of Humulus PPEs from different Shanghai areas*

To test the allergenic intensity of *Humulus* PPE, the ELISA assay was employed by using sensitized SD rat serum against *Humulus* allergens. In the experiment, the PPE of non-allergenic *Arabidopsis thaliana* pollen was used as a negative control. As shown in Fig. 5, the OD value induced by the sensitized rat serum was much higher than that of the control and blank groups. Meanwhile, the OD value of non-allergenic *Arabidopsis* PPE immune-reacted with sensitized rat serum was close to the background level (Fig. 5a). The pattern of allergenic intensity induced by the different pollen samples was WS > JD > XJH > SJ (Fig. 5c). The data of the western blot assay showed similarity with those of the ELISA assay (Appendix A Fig. S2).

2.6. *Correlation between allergenicity of Humulus pollens collected from different areas and SO_2, NO_2, O_3 and PM_{10}/$PM_{2.5}$*

Average mass concentrations of SO_2, NO_2 and PM_{10}, $PM_{2.5}$ were calculated from on-line daily monitoring data, and the allergenicity of *Humulus* pollens was expressed as the ELISA optical density at 450 nm wavelength (OD_{450nm}) value. The allergenicity of *Humulus* pollen exhibited a significant negative correlation with the mass level of PM_{10} and $PM_{2.5}$, resulting in regression coefficients (R) of -0.99 and -0.73, respectively (Table 1), suggesting that PM_{10} and $PM_{2.5}$ imposed negative effects on the intracellular allergenic protein contents in identical quantities of *Humulus* pollen. On the contrary, the mass concentration of O_3 had a close relationship with the OD_{450nm} value (R = 0.92), implying that O_3 had a positive effect on the allergenicity of the *Humulus* pollen. The gaseous pollutants NO_2 (R = -0.25) and SO_2 (R = -0.21) had no relationship with the allergenicity of *Humulus* pollen.

3. Discussion

Air pollutants have been indicated as contributing factors to the increased incidence of allergic diseases observed in recent

Table 1 – Relationship between allergenicity of *Humulus* pollens with main air pollutants in Shanghai atmosphere.

	PM_{10} (μg/m³)	$PM_{2.5}$ (μg/m³)	SO_2 (μg/m³)	NO_2 (μg/m³)	O_3 (μg/m³)	OD value
WS	68.58 ± 29.39	39.90 ± 23.85	17.43 ± 8.17	46.82 ± 20.15	98.00 ± 2.82	0.84 ± 0.21
JD	75.47 ± 29.37	38.97 ± 21.47	14.94 ± 6.47	52.21 ± 21.10	68.50 ± 21.92	0.59 ± 0.03
SJ	80.38 ± 35.71	52.37 ± 25.94	18.83 ± 8.97	47.34 ± 29.74	67.50 ± 24.74	0.45 ± 0.05
XJH	73.71 ± 28.55	41.2 ± 22.8	15.46 ± 8.97	48.62 ± 20.73	74.50 ± 13.43	0.65 ± 0.02
R	-0.99**	-0.73**	-0.21*	-0.25**	0.92**	

R: regression ecoefficiency; PM_{10}/$PM_{2.5}$: particulate matter with air dynamic diameter less than 10 or 2.5 μm; OD: optical density.
** Correlation is significant at the 0.001 level.
* Correlation is significant at the 0.05 level.

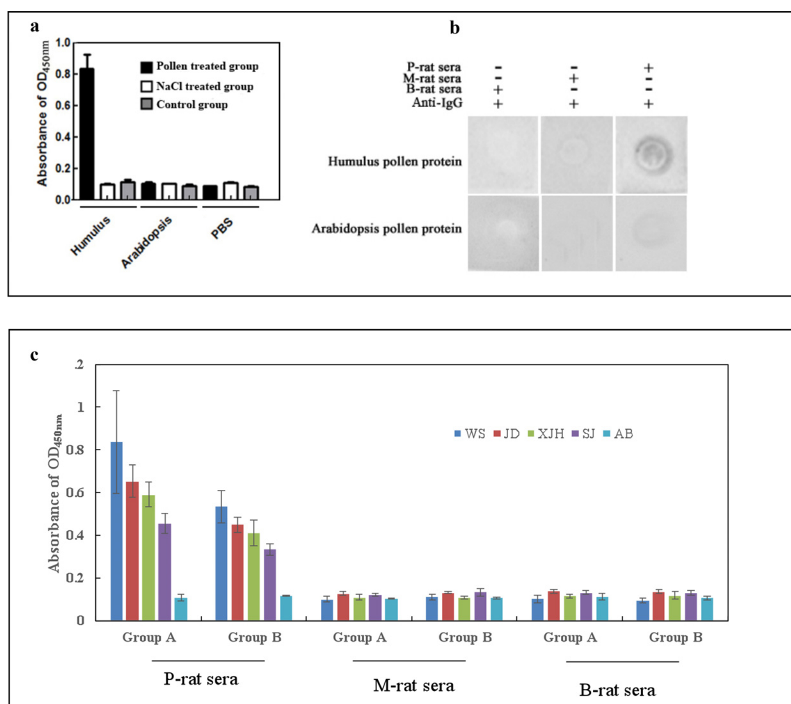

Fig. 5 – Allergenic intensity assay of *Humulus* pollen. (a) Specific immune-reactive assay of *Humulus* using enzyme-linked immunosorbent assay (ELISA); (b) Dot blot assay of pollen protein; (c) ELISA assay of pollen collected from different regions of Shanghai. P-rat sera: rats treated with pollen, M-rat sera: rats treated with NaCl; B-rat sera: the rat sera obtained from PBS treated group. AP: *Arabidopsis* protein; PBS: phosphate buffered solution; OD_{450nm}: optical density at 450 nm wavelength.

years (Aina et al., 2010). Urbanization and high levels of vehicle emissions were correlated with the increasing frequency of pollen-induced respiratory allergy, and people living in urban areas tend to be more affected by pollen-induced respiratory allergy than those who live in rural areas (Ishizak et al., 1987).

We previously reported that asthma caused by ambient pollen has led to 8.23% of Shanghai children not being able to participate in outdoor physical activities and caused 5.02% to be absent from educational services for more than two months (Lu et al., 2014). Importantly, pollen cytoplasm could release from pollen under certain humidity conditions (Wang et al., 2012), thus becoming a major source of allergen-containing micro particles (Yan et al., 2019). Up to now, the allergenicity of *Humulus* pollen in the presence of air pollutants has not been studied. Considering the possibility that different areas exhibited differential mass levels of air pollutants, we collected fresh *Humulus* pollen in several regions of Shanghai, and tried to reveal the relationship between the allergenicity of *Humulus* pollen and the main air pollutants in the Shanghai atmosphere. Our data showed that the allergenicity of *Humulus* pollen exhibited a significant negative correlation with PM_{10} and $PM_{2.5}$, resulting in regression coefficients (*R*) of -0.99 and -0.73, respectively (Table 1), suggesting that PM_{10} and $PM_{2.5}$ imposed negative effects on intracellular allergenic protein contents in the identical quantity of *Humulus* pollen. This result agreed with our previous study, which demonstrated that Ca^{2+}, NH_4^+, and SO_4^{2-} in Shanghai ambient particles could promote the breakage of *Platanus* pollen (Zhou et al., 2018), and that Ca^{2+} in Yellow Sand affected the release of allergenic pollen species of *Cryptomeria japonica* 1 (*Cry j* 1) (Wang et al. 2012).

In combination with the results from the experiments above, we deduced that the ambient particles might cause the pollen protein to be released from pollen, hence explaining the lesser amount of protein obtained from the freshly collected pollen. O_3 had a close relationship with the OD_{450nm} value ($R = 0.92$), implying that O_3 might have a positive effect on the allergenicity of the *Humulus* pollen. Previously, we reported that O_3 exposure primarily causes sulfide bond formation and enhances the allergenicity of pollen (Hong et al., 2018). Considering that the mass level of O_3 (98.00 ± 2.82 μg/m³) in the

WS atmosphere was higher than that of other areas, the data on O_3 and allergenicity therefore agreed with the result from our previous study that O_3 could enhance the allergenicity of *Humulus* pollen (Hong et al., 2018).

Buters et al. (2015) investigated the pollen allergens contained in or attached to particulate matters within 10 sites over Europe, and they found that major group 5 grass allergens (in Europe) contained in or attached to the particulate matters, including pollen grains with radius over 10 µm, decreased, whereas the released allergen attached to 2.5–10 µm particulate matters increased significantly, exhibiting good positive correlation with the humidity of the areas (Buters et al., 2015). Our ELISA results demonstrated that the remaining allergen retained in *Humulus* pollen showed a good negative correlation with $PM_{10}/PM_{2.5}$ (particulate matter with air dynamic diameter less than 10 or 2.5 µm) concentrations. On the other hand, the total protein amount in WS and JD samples was higher than that in SJ and XJH (Fig. 3a), and the allergenic intensity of the different pollen proteins showed the same pattern (WS > JD > XJH > SJ) (Fig. 5). This data strongly suggested that pollen breakage and the release of pollen intracellular contents led to the variation in allergenicity. Therefore, our results were consistent with those of Buters et al. (2015), who reported that pollen allergens would be released from pollen and spread *via* particulate matters, and that the concentration of $PM_{2.5}/PM_{10}$ and the air quality of the sampling sites could substantially influence allergen release from the *Humulus* pollen.

4. Conclusions

The biological viability of *Humulus scandens* pollen collected from four districts (WS, JD, SJ and XJH) exhibited no significant difference. *The Humulus* pollen grains exhibited a nearly globular shape and light-yellow color, and possessed a tough and smooth wall. An SDS-PAGE assay demonstrated that the *Humulus* pollen protein extract (PPE) exhibited some smearing on the SDS-PAGE gel. The sera obtained from allergic SD rats showed specific immune-reactive response against *Humulus*. A PPE Western blot assay demonstrated that the 15 KD band of *Humulus* pollen proteins showed specific immune-reactive response against sensitized rat serum and human serum. Furthermore, the pollen protein extracts from different districts of Shanghai exhibited different allergenic intensities. $PM_{10}/PM_{2.5}$ might aggravate the rupture of pollen, so that the allergen contained in the *Humulus* pollen would be released into the air and the amount of allergen retained in the *Humulus* pollen would be decreased. Although the $PM_{10}/PM_{2.5}$ levels had negative effects on the allergenicity of the pollen allergen, they should contribute to the mass level of allergenic protein in the atmosphere. O_3 had a close relationship with the allergenicity of the pollen, suggesting enhancement of allergenicity.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jes.2020.03.037.

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