

Characterization of the Physical Form of Allergenic Cry j 1 in the Urban Atmosphere and Determination of Cry j 1 Denaturation by Air Pollutants

Qingyue Wang*, Jun Morita, Xiumin Gong, Shinichi Nakamura, Miho Suzuki, Senlin Lu¹⁾, Kazuhiko Sekiguchi, Takuya Nakajima, Daisuke Nakajima²⁾ and Makoto Miwa³⁾

Graduate School of Science and Engineering, Saitama University, 255 Shimo-Okubo, Sakura-ku, Saitama City, Saitama 338-8570, Japan

¹⁾School of Environmental and Chemical Engineering, Shanghai University, Shanghai 200444, China

²⁾Research Center for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba City, Ibaraki 305-8506, Japan

³⁾Center for Environmental Science in Saitama, 914 Kamitanadare, Kisaimachi, Kitasaitama-gun, Saitama 347-0115, Japan

*Corresponding author. Tel: +81-48-858-3733, E-mail: seiyo@mail.saitama-u.ac.jp

ABSTRACT

In this study, we characterized the physical form of allergenic Cry j 1 in the urban atmosphere. Through an immunofluorescence antibody method, we showed that allergenic Cry j 1 exists as fine particles ($\leq 1.1 \mu\text{m}$). To determine Cry j 1 concentrations and its particle size distribution, we used the ELISA method to confirm that most Cry j 1 exists as fine particles in the urban atmosphere and is found at high concentrations on fine day next to rainy day. Furthermore, we evaluated Cry j 1 denaturation by using the Biacore J system based on the surface plasmon resonance (SPR) principle and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We showed that the dissociation constant (K_D) of Cry j 1 that has been exposed to urban polluted air is lower ($1.76 \times 10^{-14} \text{ M}$) than that of Cry j 1 (1.32×10^{-9} – $3.37 \times 10^{-9} \text{ M}$) of original pollen grains that has not been exposed to air pollutants. Cry j 1 turns into low molecular weight proteins by reacting with various acidic solutions. In sum, we showed that allergenic Cry j 1 exists as fine particles that can deposit in the lower respiratory tract. This finding clarifies the relationship between Japanese cedar pollinosis and air pollutants.

Key words: Japanese cedar pollinosis, Allergenic Cry j 1, Surface plasmon resonance (SPR), Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Air pollutants

1. INTRODUCTION

Pollen allergies are common throughout the world.

Grass pollen allergy in Europe, ragweed pollen allergy in America, and Japanese cedar pollinosis in Japan are known as the three major pollen allergies in the world. Japanese cedar pollinosis was reported in 1964 for the first time (Horiguchi *et al.*, 1964), and its prevalence has increased every year since 1980s due to the after-war reforestation policy. Japanese cedar pollen grains are coarse particles that measures $30 \mu\text{m}$ in diameter. It mainly deposits in the upper respiratory tract because of its size. Allergenic Cry j 1 and Cry j 2 are causative substances of Japanese cedar pollinosis and are responsible for the induction of allergic symptoms. Cry j 1 is a basic protein with a molecular weight of 41 kDa and 44 kDa. It is localized in Ubisch bodies (around $0.7 \mu\text{m}$) which are attached mainly on the surface of the Japanese cedar pollen grain (Nakamura *et al.*, 2004). Cry j 2 is also a basic protein with a molecular weight of 37 kDa. It is localized in starch granules and the pollen lining membrane inside a pollen grain, and its content is a tenth of Cry j 1 (Nakamura *et al.*, 2004).

Recently, Japanese cedar pollen grains have been thought to deposit only in the human nasal cavity and mouth but not in lower respiratory tract. However, allergenic Cry j 1 has been proposed to deposit in the lower respiratory tract (Takahashi *et al.*, 1993) and is thought to induce Japanese cedar pollinosis and asthma (Maeda, 1999). It is important to confirm physical form of airborne Cry j 1 which can deposit in the lower respiratory tract because the induction of Japanese cedar pollinosis adversely affects human health and poses as a public health concern.

In 2008, 26.5% of the Japanese population had an onset of Japanese cedar pollinosis, with cases mainly in Kanto area (33.5%) (Murayama *et al.*, 2010) of Japan. Based on these findings, we aimed to determine the relationship between Japanese cedar pollinosis and air

pollutants. The number of patients with Japanese cedar pollinosis correlates with the amount of traffic (Ishizaki *et al.*, 1987), and typical air pollutants (O_3 and NO_2) exacerbate allergy symptoms of guinea pig (Kobayashi *et al.*, 2008).

In this study, we defined the physical form of allergenic Cry j 1 found in an urban atmosphere and verified Cry j 1 fine particles which can deposit in the lower respiratory tract. Moreover, we evaluated the denaturation of Cry j 1 protein to determine the relationship between Japanese cedar pollinosis and urban air pollutants.

2. MATERIALS AND METHODS

2.1 Sampling Sites and Sample Collection

2.1.1 Sampling Locations and Periods of Airborne Pollens and Allergenic Cry j 1 Particles

Airborne pollens and allergenic Cry j 1 particles were collected at the roadside near Saitama University of Japan, representative of an urban area in the Kanto Plain of Japan. In order to calculate the amount of airborne Japanese cedar pollens, the pollens were collected on a glass slide coated with white petrolatum by using a Durham sampler (DK-1SA, Nishiseiki Co., Ltd.), a standard method in Japan. Moreover, size-segregated airborne particles of three different particle sizes ($\leq 1.1 \mu\text{m}$, $3.3 \mu\text{m}$ - $7.0 \mu\text{m}$ and $\geq 7.0 \mu\text{m}$) were collected by using an Andersen high volume air sampler (AH-600, AHV, Shibata scientific technology Co., Ltd., Japan) with the flow rate of 566 L/min for 71 hours in 2007 and for 47 hours in 2008. Pollen sampling was carried out from March 2nd-24th in 2008, and air sampling was carried out from March 5th-April 2nd in 2007 and March 2nd-24th in 2008. All size-segregated airborne particles collected on quartz fiber filters (AHQ-630 (diameter: 305 mm) and QAT-UP (205 × 255), Tokyo Dylec Corp., Japan) of the Andersen high volume air sampler were stored at -40°C .

2.1.2 Fresh Pollen Grain Collection in Various Sampling Sites

We collected fresh pollen grains from three sites: Hitachi of Ibaraki Prefecture, Chichibu of Saitama Prefecture and Hachioji of metropolitan Tokyo in Kanto Plain of Japan. In these sites, Japanese cedar (*Cryptomeria japonica*) branches with anthers were cut off from a cedar tree and stored in an airtight plastic bag. The airtight plastic bag was then shaken, releasing fresh pollen grains from the pollen perigonium on the tree branch. The sampling period was from February to March during Japanese cedar pollen dispersion period in 2008.

2.2 Determination of Physical Form of Allergenic Cry j 1 in the Urban Atmosphere

In order to verify the physical form of allergenic Cry j 1 in the urban atmosphere, we applied an immunofluorescence antibody method developed in our previous study (Wang *et al.*, 2008). The experimental procedure is given below.

The filters (1st stage, $\geq 7.0 \mu\text{m}$; 5th stage, $\leq 1.1 \mu\text{m}$) that collected airborne particles in 2007 were cut out ($\phi 5 \text{ mm}$) and put on the bottom of each well of microplate (MS-8896F, Sumitomo Bakelite Co., Ltd., Japan). Next, 100 μL of anti-Cry j 1 monoclonal antibody (clone 013, Seikagaku Biobusiness Co., Ltd., Japan, 1 : 50 diluted in phosphate buffered saline (PBS)) was added to each well of a micro-plate, and it was incubated for 2 hours at 37°C . After washing once with 250 μL PBS containing 0.05 wt% Tween 20 (polyoxyethylene sorbitan monolaurate) and twice with 250 μL PBS, the surface of each well was blocked by adding 250 μL of PBS containing 1 wt% bovine serum albumin (BSA). After incubation (2 hours at 37°C) and the washing step, 100 μL of anti-mouse IgG antibody, Fluorescein (FITC) conjugate (6.65 $\mu\text{g}/\text{mL}$ diluted in ultrapure water ($18.2 \text{ M}\Omega \cdot \text{cm}$), Jackson ImmunoResearch Laboratories, Inc.) was added to the each well. After incubation (2 hours at room temperature) and the washing step, the filters were taken out from the each well. Each filter was put on a grass slide ($76 \text{ mm} \times 26 \text{ mm}$, Matsunami Glass Ind., Ltd., Japan), and one drop of enclosure reagent (Vector Shield Mounting Medium, Vector Laboratories Inc.) was added to the each filter. After putting cover glass ($18 \text{ mm} \times 18 \text{ mm}$, Matsunami Glass Ind., Ltd., Japan) on the each filter respectively, it was observed by using a fluorescence microscope (MX6300, Meiji Techno Co., Ltd., Japan; excitation wavelength: 470 nm) equipped a CCD camera (Penguin 600CL, Pixera Co., Ltd.).

2.3 Measurement of Cry j 1 Concentration and Its Particle Size Distribution by ELISA Method

2.3.1 Sample Preparation for Measurement of Cry j 1 Concentrations and Size Distribution

The filters (1st stage, $\geq 7.0 \mu\text{m}$; 2nd stage, $3.3 \mu\text{m}$ - $7.0 \mu\text{m}$; and 5th stage, $\leq 1.1 \mu\text{m}$) that collected airborne particles in 2008 were cut out ($\phi 8 \text{ mm}$, 30 pieces) and put into centrifuge tubes (polypropylene copolymer), followed by the addition of 3 mL of pollen extracting solution (0.125 M NH_4HCO_3 , 150 mM NaCl, 3 mM EDTA, 0.005 wt% Tween 20, and 10 mM HEPES buffer solution) (Takahashi *et al.*, 2002; Takahashi *et al.*, 2001). After incubation of 24 hours at 4°C , the centrifuge tubes were shaken at 192 rpm at room tempera-

ture for 1 hour in the incubator (UNIMAX 2010, Heidolph Co. Ltd.), followed by centrifugation at 3000 rpm for 30 minutes at room temperature using the centrifuge (CN-1050, AS ONE Corp., Japan). The supernatant were collected and used for ELISA analysis. In our previous study, we determined that Cry j 1 concentrations of 3rd stage filter (2.0 μm -3.3 μm) and 4th stage filter (1.1 μm -2.0 μm) were very low. Therefore, we didn't measure Cry j 1 concentration of these filters in this study.

2. 3. 2 Analytical Procedure of ELISA Method

ELISA (enzyme-linked immunosorbent assay) method was employed to measure allergenic Cry j 1 concentrations and its particle size distributions. Briefly, 100 μL of anti-Cry j 1 monoclonal antibody (clone 013, Seikagaku Biobusiness Co., Ltd., Japan, 1 : 50 diluted in PBS) was added to each well of a micro-plate and it was incubated for 2 hours at 37°C. After a washing step of once with 250 μL PBS containing 0.05 wt% Tween 20 and twice with 250 μL PBS, the surface of each well was blocked by adding 250 μL of PBS containing 1 wt% BSA. After a washing step, ELISA sample solution and standard solution containing Cry j 1 (Japanese Cedar Pollen Allergen Cry j 1, Purified, Seikagaku Biobusiness Co., Ltd., Japan) at 0.5, 1, 2, or 5 ng/mL diluted in PBS was added to each well. After incubation of 2 hours at 37°C and a washing step, 100 μL of anti-Cry j 1 monoclonal antibody with horseradish peroxidase (HRP) conjugate (clone 053, Seikagaku Biobusiness Co., Ltd., Japan, 1 : 1,000 diluted in PBS containing 0.1 wt% BSA) was added to each well. After incubation of 2 hours at 37°C and a washing step, 100 μL of *o*-phenylenediamine solution (0.5 mg/mL diluted in 0.03 M citric acid/0.07 M phosphoric acid buffer solution) as a chromogenic substrate was added to each well. The chromogenic reaction was stopped after 30 min by adding 100 μL of 1 M H_2SO_4 to each well. The absorbance was measured at 492 nm with 630 nm as the reference wavelength by the micro-plate reader (MP-1000, Microtech Co., Ltd., Japan).

2. 4 Measurement of Association and Dissociation Rate Constants between Cry j 1 Antigens and Anti-Cry j 1 Monoclonal Antibody by a Biacore J System

2. 4. 1 Cry j 1 Sample Preparation for Biacore J Measurement

Cry j 1 components were extracted from the fresh pollen grains collected in three sites: Hitachi of Ibaraki Prefecture, Chichibu of Saitama Prefecture and Hachioji of metropolitan Tokyo. 500 mg of Japanese cedar fresh pollen grains were put into the centrifuge tube,

followed by the addition of 50 mL of pollen extracting solution. After incubation at room temperature for 3 hours in the incubator (UNIMAX 2010, Heidolph), the centrifuge tube was centrifuged at 15,000 rpm for 10 minutes by the centrifuge (CN-1050, AS ONE Corp., Japan). The supernatant (Cry j 1 samples in fresh pollen grains that were not exposed to air pollutants) were collected for Biacore J measurement as fresh pollen extracts.

The filter (5th stage, $\leq 1.1 \mu\text{m}$) that collected airborne particles in 2008 was cut out ($\phi 8$ mm, 30 pieces) and stored in centrifuge tube. 3 mL of pollen protein extracting solution was added, followed by an incubation of 24 hours at 4°C to extract the Cry j 1 protein. The centrifuge tube were then shaken at 192 rpm at room temperature for 1 hour in the incubator (UNIMAX 2010, Heidolph) and centrifuged at 3,000 rpm for 30 minutes at room temperature using the centrifuge (CN-1050, AS ONE Corp., Japan). The supernatant in the tube was collected and used for Biacore J measurement of airborne Cry j 1 as filter extract that was exposed to urban polluted air.

All of these samples mentioned above were prepared for determination of the association and dissociation rate constants between allergenic Cry j 1 in and anti-Cry j 1 monoclonal antibody. As a standard sample for reference, the purified Cry j 1 (Japanese Cedar Pollen Allergen Cry j 1, Purified, Seikagaku Biobusiness Co., Ltd., Japan) was also used in the present study.

2. 4. 2 Measurement Principle and Analytical Procedure of the Biacore J System Based on Surface Plasmon Resonance

We determined Cry j 1 concentrations and association and dissociation rate constants (k_a , k_d , respectively) between Cry j 1 antigens and anti-Cry j 1 monoclonal antibody by using the Biacore J system (GE Healthcare Japan Co., Ltd., Japan) based on the surface plasmon resonance (SPR) principle.

The measurement principle of Cry j 1 concentrations and dissociation constants (K_D , $K_D = k_d/k_a$) between Cry j 1 antigens and anti-Cry j 1 monoclonal antibody based on the SPR principle is shown in Fig. 1. As anti-Cry j 1 monoclonal antibody are immobilized on the surface of a sensor chip, the refractive index at the interface between the sensor chip surface and a solution flowing over the surface changes, altering the angle at which reduced-intensity polarized light is reflected from a supporting glass plane. The change in angle, caused by binding or dissociation of anti-Cry j 1 monoclonal antibody from the sensor chip surface, is proportional to the mass of bound material and is recorded in a sensor gram. Next, when a solution containing Cry j 1 antigen is passed over the sensor chip surface, the sensor gram shows an increasing response as mol-

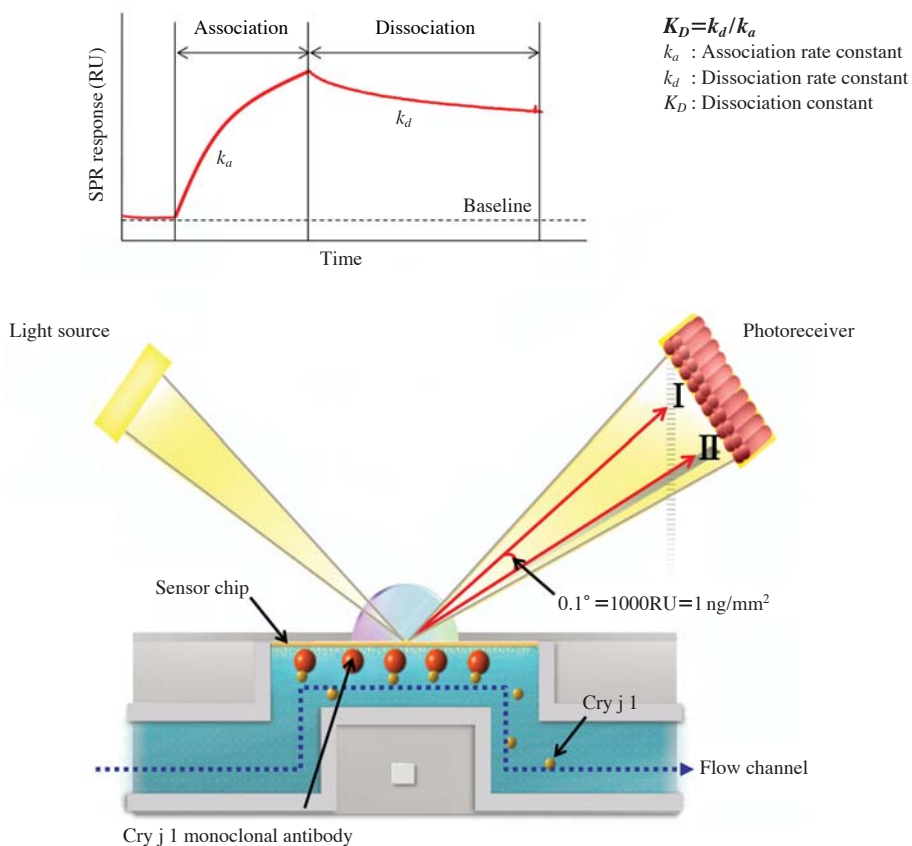


Fig. 1. Measurement principle of Cry j 1 concentrations and dissociation constant between Cry j 1 antigens and anti-Cry j 1 monoclonal antibody based on the surface plasmon resonance principle.

ecules interact. The response remains constant if the interaction reaches equilibrium. When the solution containing Cry j 1 antigen is replaced by the buffer, the response decreases as the interaction partners dissociate. Complete profiles of recognition, binding and dissociation are generated in real time. From these profiles, data such as specificity, affinity, kinetic behavior and Cry j 1 concentration can be determined.

The analysis of the Biacore J system is as follows: First, 100 μ L of N-ethyl-N'-(3-dimethylaminopropyl) carbodiimidehydrochloride solution and 100 μ L of N-hydroxysuccinimide solution were added to a micro-tube and subsequently shaken. The mixed solution was injected into an injection port of the Biacore J system and flowed for 6 minutes to activate its sensor chip (CM 5, GE Healthcare Japan Co., Ltd., Japan) surface. Next, anti-Cry j 1 monoclonal antibody (clone 013, Seikagaku Biobusiness Co., Ltd., Japan) solution (1 : 50 diluted in pH 5.0 sodium acetate solution) as a ligand solution was injected into the injection port and flowed for 6 minutes to immobilize the ligands on the sensor chip surface. 1 M ethanolamine hydrochloride

solution (pH=8.5) as a blocking reagent was next injected into the injection port and flowed for 6 minutes. Lastly, sample solution was injected into the injection port and flowed for 2 minutes. All flow rates were 30 μ L/min, and HBS-EP (BR-1001-88, GE Healthcare Japan Co., Ltd., Japan) was used as a buffer solution. 10 mM glycine-HCl solution (pH 2.0) was used as a re-claimed solution of the sensor chip.

We prepared the filter extract and four kinds of extracts using fresh pollen extracts for three sampling sites. Four kinds of fresh pollen extracts were one original extract (supernatant solution) and three others made double dilution, three time dilution and four time dilution, separately. And then, Cry j 1 concentrations in the filter extract and four kinds of fresh pollen extracts were measured respectively. The association and dissociation rate constants between Cry j 1 and anti-Cry j 1 monoclonal antibody were calculated based on four representative averaged data sets of Cry j 1 concentrations for each sampling site by BIAevaluation software (GE Healthcare Japan Co., Ltd., Japan).

2. 5 Measurement of Change in Molecular Weight of Cry j 1 Exposed Various Acidic Solutions by SDS-PAGE

2. 5. 1 Sample Preparation for SDS-PAGE Measurement

40 mL of pollen protein extracting solution was added to 3 g of fresh Japanese cedar pollen grains, followed by an incubation of 24 hours at 4°C to extract Cry j 1 protein. In order to denature Cry j 1 chemically, 5 mL of each supernatant solution prepared only from Japanese cedar pollen grains was exposed to representative solution simulated acidic air pollutants that include 2 mL of 45 mM peroxyxynitrite solution (P332, Dojindo Corporate Headquarters, Japan), 2 mL of 1 M nitric acid solution (140-04016, Wako Pure Chemical Industries Ltd., Japan), 2 mL of 1 M sulfuric acid solution (198-09595, Wako Pure Chemical Industries Ltd., Japan), and 2 mL of 1 M hydrogen peroxide solution (080-01186, Wako Pure Chemical Industries Ltd., Japan) respectively. The acidic components in the simulated solutions can be those caused from acidic gases of primary emission (such as NO_x and SO₂) and secondary photochemical formed acids (such as HNO₃, H₂SO₄, O₃ and H₂O₂). The mixture was then shaken at 192 rpm at room temperature for 1 hour in the incubator (UNIMAX 2010, Heidolph), followed by centrifugation at 6,000 rpm for 30 minutes at room temperature using the centrifuge (CN-1050, AS ONE Corp., Japan). The supernatants were then prepared for SDS-PAGE measurement of Japanese cedar pollen grains.

2. 5. 2 Analytical Procedure of a SDS-PAGE

Prepared SDS-PAGE samples were centrifuged at 6,000 rpm for 30 minutes at room temperature (CN-1050, AS ONE Corp., Japan). After sample supernatants were treated with EzApply (AE-1430, ATTO Corp., Japan), they were separated by SDS-PAGE. Proteins were detected by using the EzStain Silver Kit (AE-1360, ATTO Corp., Japan). This has a higher sensitivity compared with the Coomassie brilliant blue staining. EzStandard Prestain Blue (AE-1450, ATTO Corp., Japan) was used as the molecular weight marker (β -galactosidase: 114 kDa, serum albumin: 84.7 kDa, ovalbumin: 47.3 kDa, carbonic anhydrase: 31.3 kDa, trypsin inhibitor: 25.7 kDa, lysozyme: 17.4 kDa).

3. RESULTS AND DISCUSSION

3. 1 Verification of Physical Form of Allergenic Cry j 1 Existence in the Urban Atmosphere

Allergenic Cry j 1 in the urban atmosphere has been suggested to deposit in the lower respiratory tract and

induces Japanese cedar pollinosis asthma. To determine the physical form of Cry j 1 existence in the urban atmosphere, we applied an immunofluorescence antibody method that used anti-Cry j 1 monoclonal antibody and anti-mouse IgG, HRP conjugate.

Micrographs of quartz fiber filter of an Andersen high volume air sampler that carried out the immunofluorescence antibody treatment are shown in Fig. 2. Luminescent spots of green fluorescence luminescence show the presence of Cry j 1, suggesting Cry j 1 is not only present on Japanese cedar pollen surface (a') but also exists as fine particles ($\leq 1.1 \mu\text{m}$) (b'). Since fine particle ($\leq 1.1 \mu\text{m}$) can deposit in the lower respiratory tract (Hatch, 1961), our finding support the hypothesis that allergenic Cry j 1 fine particles can deposit in the lower respiratory tract to induce Japanese cedar pollinosis asthma.

3. 2 Cry j 1 Concentrations and Its Particle Size Distributions in the Urban Atmosphere

Since allergenic Cry j 1 exists as fine particles in the urban atmosphere as mentioned above, we next determined Cry j 1 concentrations and its particle size distributions in the urban atmosphere by ELISA. Airborne Cry j 1 concentrations, its particle size distributions and Japanese cedar pollen counts from March 2nd to 24th in 2008 are shown in Fig. 3. Allergenic Cry j 1 was detected at high concentrations in the range of particle diameter less than 1.1 μm and was hardly detected in the particle sizes from 3.3 μm to 7.0 μm . This finding confirms that most Cry j 1 exist as fine particles ($\leq 1.1 \mu\text{m}$) in the urban atmosphere. Fine particles are likely reacting with urban air pollutants and denaturing chemically because they have a long detention time. It is important to investigate the denaturation of Cry j 1 as fine particles ($\leq 1.1 \mu\text{m}$).

According to our previous study (Wang *et al.*, 2008), it is usually found that there are the daily variation delays in the peaks of allergenic Cry j 1 concentrations at the high levels were observed during 1 or 2 sunny days after rainfall comparing with the peaks of airborne parent Japanese cedar pollen counts. In this study, we also showed that there are the poor correlations between Cry j 1 concentrations and the pollen counts. The highest Cry j 1 concentrations were detected between March 20th and 21st, and the lowest ones between March 18th and 19th. Rainfall was observed between March 19th and 20th, and clear sky was only observed on March 21th. We have found that Cry j 1 concentrations were observed at low levels on rainy day but at high concentrations on fine day next to rainy day after the peaks of the airborne pollen counts. It is means that pollen grains and allergenic daughter particles on the

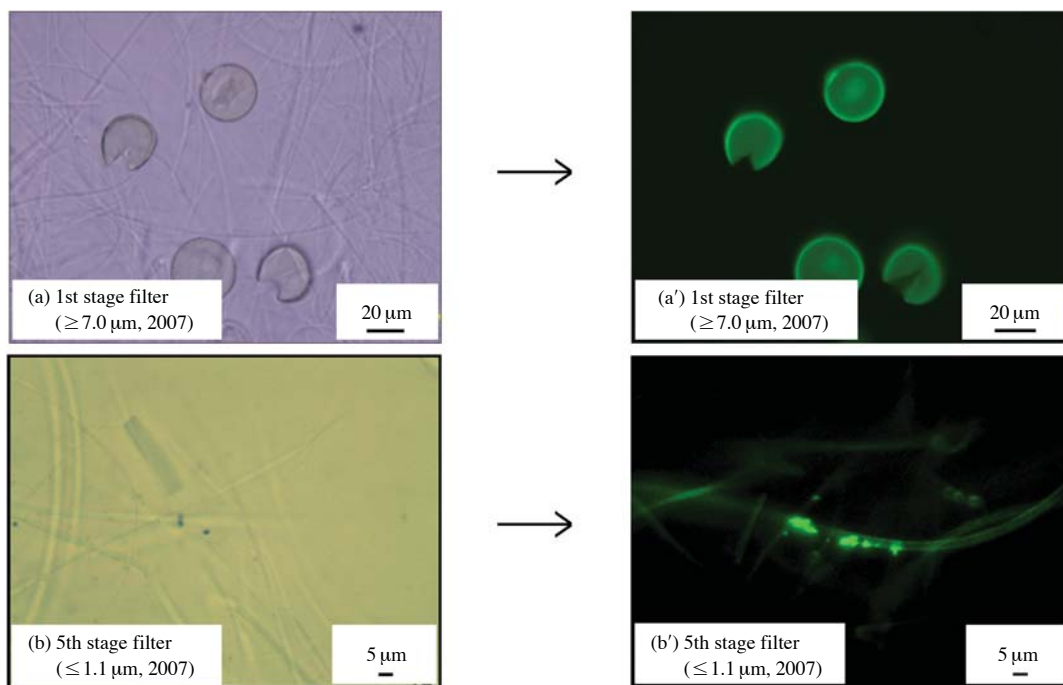


Fig. 2. Micrographs of quartz fiber filter of an Andersen high volume air sampler that carried out the immunofluorescence antibody treatment. (a): 1st stage filter ($\geq 7.0 \mu\text{m}$, 2007), (b): 5th stage filter ($\leq 1.1 \mu\text{m}$, 2007). (Left micrographs: before B excitation light was irradiated; right micrographs: after B excitation light was irradiated).

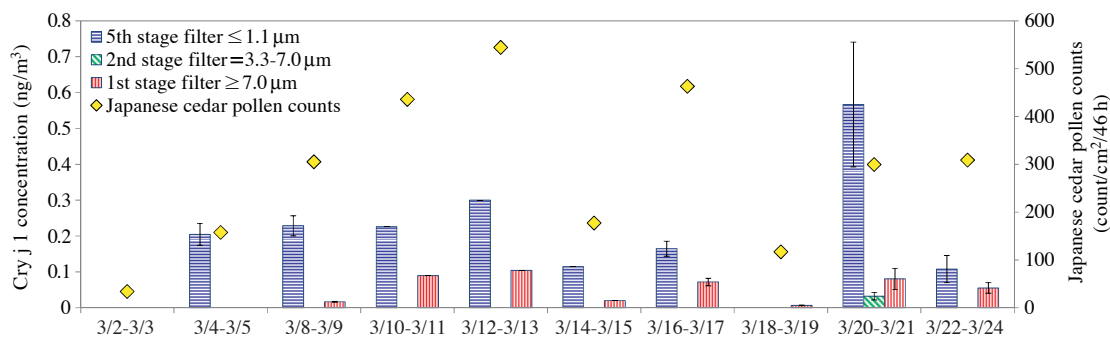


Fig. 3. Airborne Cry j 1 concentration (n=6), its particle size distribution and Japanese cedar pollen counts from Mar. 2nd to 24th in 2008.

asphalt could be resuspended by wind and heavy traffic volumes in urban area after rainfall. Currently, the information of Japanese cedar pollen counts is provided for patients with Japanese cedar pollinosis as countermeasure of Japanese cedar pollinosis. From now on, it is necessary to offer these patients not predicted Japanese cedar pollen counts but also predicted levels of Cry j 1 because Japanese cedar pollinosis is mainly caused by daughter allergenic Cry j 1 content contained in the parent pollen grains. These levels could also be determined by investigating the correlations bet-

ween allergenic Cry j 1 concentrations and other meteorological data, because Cry j 1 concentrations do not only correlate with Japanese cedar pollen counts.

3.3 Association and Dissociation Rate Constants between Airborne Cry j 1 Antigens and Anti-Cry j 1 Monoclonal Antibody

Cry j 1 coarse particles (1st stage, particle sizes $\geq 7.0 \mu\text{m}$) are difficult to react with air pollutants because they have a short detention time in atmosphere. Fur-

Table 1. Cry j 1 concentrations, association rate constant, dissociation rate constant, dissociation constant between Cry j 1 antigens and anti-Cry j 1 monoclonal antibody.

Cry j 1 samples	k_a (1/Ms)	k_d (1/s)	K_D (M)	Cry j 1 concentration ($\mu\text{g/mL}$)
Cry j 1 that weren't exposed to air pollutants (fresh pollen in Saitama)	4.76×10^5	8.51×10^{-4}	1.79×10^{-9}	6.29 ± 0.23 (fresh pollen in Saitama)
Cry j 1 that weren't exposed to air pollutants (fresh pollen in Ibaraki)	2.44×10^6	3.21×10^{-3}	1.32×10^{-9}	6.23 ± 0.26 (fresh pollen in Ibaraki)
Cry j 1 that weren't exposed to air pollutants (fresh pollen in Hachioji)	1.76×10^5	5.92×10^{-4}	3.37×10^{-9}	6.58 ± 0.58 (fresh pollen in Hachioji)
Purified Cry j 1 (standard sample)	3.00×10^5	6.17×10^{-4}	2.06×10^{-9}	Standard Cry j 1
Cry j 1 exposed to urban polluted air (5th stage filter ($\leq 1.1 \mu\text{m}$, 2008))	2.22×10^8	3.90×10^{-6}	1.76×10^{-14}	—

thermore, allergenic Cry j 1 contents contained in the cedar pollen grains of the perigonium were seldom influenced by urban air pollutants. Therefore, in our study, we only paid attention to the denaturation of Cry j 1 fine particles ($\leq 1.1 \mu\text{m}$) on 5th stage.

Cry j 1 fine particles ($\leq 1.1 \mu\text{m}$) are likely to react with air pollutants and become denatured because they have a long detention time in atmosphere. In order to investigate the denaturation of Cry j 1 in the urban polluted air, we calculated association and dissociation rate constants between Cry j 1 antigens and anti-Cry j 1 monoclonal antibody by using the Biacore J system based on the SPR principle. Cry j 1 concentrations, association rate constants, dissociation rate constants and dissociation constants between Cry j 1 antigens and anti-Cry j 1 monoclonal antibody are shown in Table 1. Cry j 1 concentrations in the pollens collected in various sites (2.1.2) were almost constant (from 6.29 to 6.58 $\mu\text{g/mL}$), and the dissociation constants ($K_D = k_d/k_a$) calculated from association rate constants (k_a) and dissociation rate constants (k_d) of Cry j 1 extracted from the fresh pollens collected in various sites were also nearly constant ($K_D = 1.79 \times 10^{-9}$ – 3.37×10^{-9} M). These constants are similar to one of purified Cry j 1 ($K_D = 2.06 \times 10^{-9}$ M). These constants can be the representative results of fresh pollens in each site. However, the constant of Cry j 1 extracted from the filter (5th stage, particle sizes $\leq 1.1 \mu\text{m}$) that collected airborne particles in 2008 was different from those of Cry j 1 contained in fresh pollen in each site that weren't exposed to air pollutants. Dissociation constant (K_D) of Cry j 1 exposed to urban polluted air is very lower (1.76×10^{-14} M) than those of Cry j 1 contained in fresh pollen that weren't exposed to air pollutants given in Table 1. This evidence showed that the bimolecular interaction between allergenic Cry j 1 and anti-Cry j 1 monoclonal antibody becomes very strong and difficult to be dissociated. We thus propose that airborne allergenic Cry j 1 fine particles are denatured by reac-

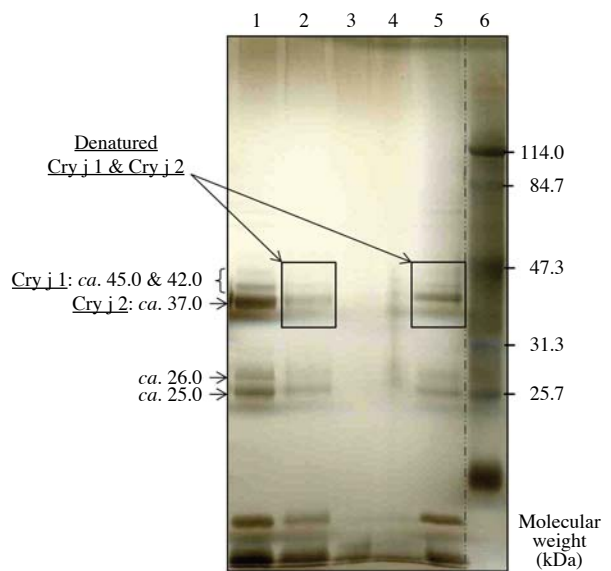


Fig. 4. SDS-PAGE profile of Cry j 1 extracts exposed to various acidic solutions. Line 1: Cry j 1 extract, Line 2: Cry j 1 + Peroxynitrite aq, Line 3: Cry j 1 + HNO_3 aq, Line 4: Cry j 1 + H_2SO_4 aq, Line 5: Cry j 1 + H_2O_2 aq, Line 6: Molecular weight marker (β -galactosidase: 114 kDa, serum albumin: 84.7 kDa, ovalbumin: 47.3 kDa, carbonic anhydrase: 31.3 kDa, trypsin inhibitor: 25.7 kDa, lysozyme: 17.4 kDa).

ting with urban air pollutants.

3. 4 SDS-PAGE Profile of Cry j 1 Extract Exposed to Various Acidic Solutions

To evaluate Cry j 1 denaturation by gaseous air pollutants caused by primary emission and secondary photochemical formation, we used SDS-PAGE to measure changes in the molecular weight of Cry j 1 induced by various acidic solutions (Fig. 4).

Protein bands corresponding to Cry j 1 protein were not detected in Line 3 (Cry j 1 + HNO_3 aq) and Line 4 (Cry j 1 + H_2SO_4 aq). This is because peptide bindings

of Cry j 1 protein were broken to peptide unit by reacting with strong acids (HNO₃ aq, H₂SO₄ aq). Therefore, Cry j 1 may be degrade down to low molecular weight peptide subunits and not be detectable by SDS-PAGE. On the other hand, protein bands corresponding to Cry j 1 protein were of lower molecular weight in Line 2 (Cry j 1 + peroxynitrite aq) and Line 5 (Cry j 1 + H₂O₂ aq) than Line 1 (Cry j 1 extract alone). This is because the peptide bindings were not broken completely due to the weaker acids (peroxynitrite aq, H₂O₂ aq). Our findings suggest that Cry j 1 reacts with acidic air pollutants and degrade into lower molecular weight proteins due to broken peptide binding.

4. CONCLUSIONS

In this study, we have shown that allergenic Cry j 1 fine particles ($\leq 1.1 \mu\text{m}$) exist in the urban atmosphere, supporting the hypothesis that Cry j 1 fine particles can deposit in the lower respiratory tract to induce Japanese cedar pollinosis asthma. Then, we firstly determined that airborne Cry j 1 fine particles are denatured by reacting with urban air pollutants because Cry j 1 exposed to urban polluted air showed higher dissociation constant (K_D) by using SPR method. It also means that Cry j 1 exposed to urban polluted air can develop very strong bimolecular interaction with anti-Cry j 1 monoclonal antibody. Lastly, Cry j 1 is degraded to peptide units through reacting with acidic air pollutants.

Given our findings, it is therefore critical to investigate Cry j 1 denaturation caused by urban air pollutants and the adverse effects of denatured Cry j 1 on human health.

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