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Field Investigation on Modification of Japanese Cedar Pollen Allergen in Urban Air-Polluted Area

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Abstract**—**Cry j 1 is a causative substance of Japanese cedar pollinosis, and it may deteriorate by Cry j 1 invasion to a lower respiratory tract. We observed airborne particles containing Cry j 1 by an immunofluorescence technique using a fluorescence microscope, and we clarified that Cry j 1 exist as aggregates of airborne fine particles (< 1.1 μm) in the urban atmosphere. Airborne Cry j 1 may react with air pollutants and be denature to a substance deteriorated Japanese cedar pollinosis. Therefore, we applied a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate a Cry j 1 reacted with various air pollutants by liquid phase reaction, and calculated kinetics constants of Cry j 1 extracted from pollens collected in various sites and airborne fine particles containing Cry j 1 by using a surface plasmon resonance (SPR) method. As a result, it is suggested that Cry j 1 may be denatured by air pollutants during the transportation to the urban atmosphere.

Keywords**—**Cry j 1, Japanese cedar pollinosis, SDS-PAGE, SPR

INTRODUCTION

FOR the first time, Japanese cedar pollinosis was reported in 1964 [1]. Recently, the Japanese of over 26% are suffering from Japanese cedar pollinosis [2], and it can say to be the national illness. Especially, prevalence of Japanese cedar pollinosis is 39.6 % in Kanto area in Japan and, the influence of air pollutants is feared. Moreover, since the increase in number of hay fever patients [3], the hay fever patient's lowering of the age [4] and the deterioration of asthma symptom [5] are also shown in the recent years, it becomes a big social problem as a present disease, and the immediate remedy is needed.

The causative substances of Japanese cedar pollinosis are Japanese cedar pollen allergens, and two kinds are known as main allergens. One is a basic protein called as Cry j 1 that molecular weight is *ca.* 41 kDa and 44 kDa (isoelectric point as 8.9 and 9.2), and Cry j 1 is localized in Ubisch bodies (diameter *ca.* 0.7 μm) attached mainly on the surface of Japanese cedar pollen (diameter *ca.* 30 μm) [6].

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Another is called as Cry j 2 that is a basic protein of molecular weight *ca.* 37 kDa (isoelectric point as 9.5) [6]. Cry j 2 is localized in the starch granules and pollen lining membrane inside the pollen, and its content is tenth part of the amount of Cry j 1[6].

Japanese cedar pollen has been thought to invade only to human nasal cavity and mouth and that there was no inhalation to a lower respiratory tract. But, it was suggested that the airborne fine particles containing Cry j 1 can invade into a lower respiratory tract recently. Some researchers found and reported that the Ubisch bodies containing respirable Cry j 1 exfoliated from the pollen surface, and they proposed the possibility of deterioration of pollen asthma [7]. Therefore, it is very important to investigate the behavior and morphology of airborne Japanese cedar pollen and airborne fine particles containing Cry j 1 in the urban polluted atmosphere of Japan.

Cry j 1 is a basic protein which has sixteen tyrosine residues (NCBI Entrez protein database, BAB86287), the tyrosine residue that has a benzene ring may generate the nitration reaction easily compared with other amino acid residues. Protein containing 3-nitrotyrosine residues evade central immune tolerance and cause robust immune reaction [8] and shows higher immunogenicity in vivo [9]. Moreover, nitration rendered the allergen bet v 1a which is a birch pollen allergen protein more immunogenic in vivo [9]. High concentration of air pollutants are caused by the heavy traffic in Kanto area of Japan. A benzene ring contained tyrosine residue may react with hydroxyl radical or NO_3 radical which comes from exhaust gas to form tyrosine intermediate, and it may react with typical air pollutant NO_2 to form nitrated tyrosine [10]. Similarly, protein and Cry j 1 containing tyrosine residues may also react with NO_2 to form nitrated protein and nitrated Cry j 1. Therefore, allergy response is promoted by nitrated protein or nitrated Cry j 1 invasion to a lower respiratory tract, and it is assumable that prevalence of Japanese cedar pollinosis increased in Kanto area of Japan.

In this study, we observed the airborne particles containing Cry j 1 by an immunofluorescence technique using a fluorescence microscope. Moreover, we evaluated the Cry j 1 denaturation by using SDS-PAGE and SPR method because Cry j 1 protein may chemically denature by reacting with the air pollutants.

MATERIALS AND EXPERIMENTAL METHODS

A. Sampling Locations and Periods

1. Sampling Locations

Airborne pollens and fine particles containing Cry j 1 were collected at Kanto area of Japan. We chose the general urban area (Cooperative Research Center of Saitama University (35.862N, 139.608E altitude *ca*. 8 m, height ca. 10 m)) and two roadsides as the sampling locations. Route 57 (35.860N, 139.606E) is located in the ca. 400 m south-southwest and route 463 (35.865N, 139.607E) is located in the ca. 300 m north from the urban area. These sampling locations showed in Fig 1.

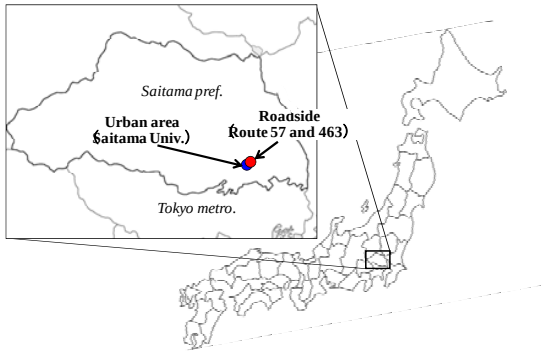

Fig. 1 Sampling sites located in the urban area of Kanto, Japan.

2. General Urban Area and Roadside Sampling Period in 2007

The consecutive samplings were carried out in the urban area and roadside (route 57). Size-segregated air samples in three different sizes (< 1.1 μm, 3.3 μm ~ 7.0 μm and > 7.0 μm) were collected by using two Andersen high volume samplers (AH-600, AHV, Shibata, Japan, **Fig. 2**) with the flow rate of 566 L/min for 71 hours. Sampling period was from 5th Mar to 29th Mar, 2007.

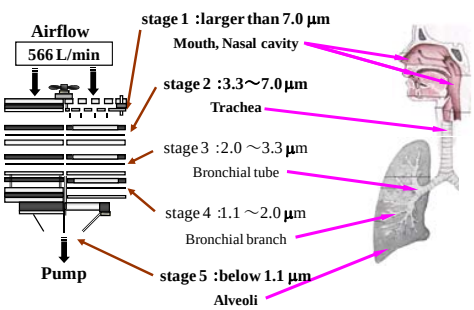

Fig. 2 Principle of an Andersen high volume sampler.

3. Two Roadsides Sampling Period in 2008

The consecutive samplings were carried out in two roadsides (route 463, route 57). Size-segregated air samples in three different sizes (< 1.1 μm, 3.3 μm ~ 7.0 μm and > 7.0 μm) were collected by using two Andersen high volume samplers with a flow rate of 566 L/min for 47 hours from 11th Feb to 22th Mar, 2008 same as the 2007 sampling shown in Fig. 2.

All the size-segregated air samples collected on the quartz fiber filters (AHQ-630 (diameter is 305 mm) and QAT-UP (205 × 255), Tokyo Dylec Corp.) of the Andersen high volume sampler were stored at -40 degrees Celsius.

4. Collection of Pollen in Various Sites

For the SPR measurement, we collected pollen samples in the three sites (Hitachi, Ibaraki; Chichibu, Saitama; Tama, Tokyo). Sampling period was from February to March, 2008. The anther of Japanese cedar branch was cut off and put into an airtight plastic bag. Then, the airtight plastic bag which held with an anther of Japanese cedar branch was shaken, and pollens were released from the branch as a result.

B. Morphology Verification of Respirable Cry j 1 Particles

1. Morphology Verification of Respirable Cry j 1 Particles by An Immunofluorescence Technique with A Fluorescence Microscope

Our study applied the immunofluorescence technique with a fluorescence microscope to verify existence form of Cry j 1 particles. Cry j 1 contained in airborne fine particles can be visualized by the specific bond between Cry j 1 antigen and Cry j 1 antibody, and performed B excitation light irradiation (wavelength 470 nm) and fluorescence detection. Especially, it was confirmed by observing Japanese cedar pollen grains and its allergen collected as the airborne particles below 1.1 μm with the developing method of an immunofluorescence technique. The experimental principle showed in Fig.3 and its procedure is given below.

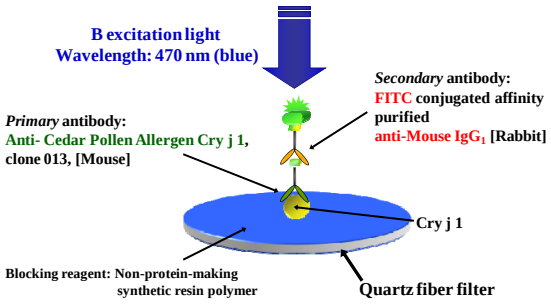

Fig. 3 Principle of an immunofluorescence technique.

The filters collected air samples were cut out (5 mmϕ) and put on the bottom of microplate wells (MS-8896F, Sumitomo Bakelite, Japan). Next, 100 μL of anti-Cry j 1 monoclonal antibody (clone 013, Seikagaku Biobusiness, Japan) was added to the each well. After incubation (2 hours at 37 degrees Celsius) and aspirating the solution, the each well was washed by 100 μL of phosphate buffered saline (PBS) containing Tween 20 (Polyoxyethylene Sorbitan Monolaurate; Surface active agent) and 100 μL of PBS (Washing 1 times). Next, 250 μL of PBS containing 1 % bovine serum albumin (BSA) was

added to the each well. After incubation (2 hours at 37 degrees Celsius), aspirating the solution and washing step (1 time), 100 μL of FITC (Fluorescein isothiocyanate) conjugated anti-mouse IgG antibody (Jackson immunoresearch Laboratories, Inc.) was added to the each well. After further incubation (2 hours at room temperature), aspirating the solution and washing step (1 time), the filters carried out the immunofluorescence process were taken out from each well. The filters were put on the grass slide, and one drop of enclosure agent (Vector Shield Mounting Medium, Vector Laboratories Ltd.) was added to the filters. After putting cover glass on the filters, they were observed by fluorescence microscope (MX6300, Meiji Techno Co. Ltd.) of incident-light. The evidence of Cry j 1 existence in the airborne fine particles (< 1.1 μm) could be confirmed with this immunofluorescence technique.

C. Investigation of Cry j 1 Modification by SDS-PAGE

1. Japanese Cedar Pollen Extracts Exposure to Simulated Pollutants

40 mL of the pollen extracting solution (0.125 M NH_4HCO_3 solution containing 150 mM NaCl, 3 mM EDTA, 0.005 wt % Tween 20, and 10 mM HEPES buffer solution [11], [12]) 40 mL was added to 3 g of Japanese cedar pollen and it was stored for 24 hours at 4 degrees Celsius. 5mL of the supernatant solution was exposed to 45 mM Peroxynitrite solution (Dojindo Corporate Headquarters, P332) 2 mL (90 μmol), 1 M nitric acid solution (140-04016, Wako Pure Chemical Industries, Japan) 2 mL (2 mmol), 1 M sulfuric acid solution (198-09595, Wako Pure Chemical Industries, Japan) 2 mL (2 mmol), and 1 M hydrogen peroxide solution (080-01186, Wako Pure Chemical Industries, Japan) 2 mL (2 mmol) as an environmental pollutants respectively. The mixed solutions were shaken at 192 rpm at the room temperature for 1 hour (UNIMAX 2010, Heidolph). Then, the centrifugation was carried out at 6000 rpm for 30 minutes at the room temperature (CN-1050, AS ONE, Japan), and the supernatant solutions were prepared as the samples for evaluating modification of pollen proteins. These samples were separated by using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by the silver staining method.

We measured ionic components in the air samples collected on the filters of an Andersen high volume sampler (12th-16th Mar, 2008). The minimum concentrations of NO_3^- and SO_4^{2-} in $PM_{1.1}$ samples were 0.77 μg/m^3 and 1.05 μg/m^3 respectively. If pollen proteins are exposed to NO_3^- or SO_4^{2-} on the filters of an Andersen high volume sampler under the same conditions (for 47 hours with a flow rate of 566 L/min), NO_3^- and SO_4^{2-} concentrations are comparable to 20 mmol. Therefore, it find that NO_3^- and SO_4^{2-} concentrations applied in the present study are tenth part of those concentrations collected with an Andersen high volume sampler. Since the Ubisch bodies that elutes from pollen surface by precipitation may react with air pollutants in environment, a liquid phase reaction was applied in the present study. During the air sampling of 12th-14th Mar, 2008, NO_3^- and SO_4^{2-} concentrations in the air samples collected on the filters of an Andersen high volume sampler were 11.34 μg/m^3 and 10.70 μg/m^3 respectively. Pollutants concentrations used in the present study corresponded to exposure for 2 hours (case of NO_3^-) and 2.5 hours (case of SO_4^{2-}) in precipitation 0.6 L (case of maximum precipitation was 19 mm on 25th Mar, 2007). In addition, hydrogen peroxide solution used as the same concentration of NO_3^- and SO_4^{2-}.

2. Extraction of Cry j 1 from Air Samples

The filters that collected air samples (particle sizes : > 7.0 μm and < 1.1 μm, sampling period and site: from 10:00 a.m. of 12th Mar, 2008 to 9:00 a.m. of 14th Mar, 2008 at Route No. 463) with an Andersen high volume sampler were cut out (8 mmϕ, 30 pieces) and put into the centrifuge tubes. 3 mL of Pollen extracting solution was added to the centrifuge tubes, and they were stored for 24 hours at 4 degrees Celsius. Then, the shaking was carried out at 192 rpm at the room temperature for 1 hour (UNIMAX 2010, Heidolph), and centrifugation was carried out at 3000 rpm for 30 minutes at the room temperature (CN-1050, AS ONE, Japan). The supernatant solutions were prepared as the samples for evaluating modification of pollen proteins (allergens).

3. Analytical Procedure of SDS-PAGE

Prepared samples (Materials and Experimental Methods C. 1, C. 2) were centrifuged at 6000 rpm for 30 minutes at the room temperature (CN-1050, AS ONE, Japan). After EzApply (AE-1430, ATTO, Japan) process was carried out for the supernatant solutions, they were separated by SDS-PAGE. The detection of proteins was carried out by using EzStain Silver Kit (AE-1360, ATTO, Japan) which is a high sensitivity method compared with a Coomassie brilliant blue staining. EzStandard Prestain Blue (AE-1450, ATTO, Japan) was used as a 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).

D. Evaluation of Intermolecular Interaction between Cry j 1 Antigen and Cry j 1 Antibody

1. Preparation of Samples

Cry j 1 was extracted from pollens collected in various sites (Materials and Experimental Methods A. 4). 500 mg of Japanese cedar pollen was added to 50 mL of pollen extracting solution. After 3 hours, the centrifugation was carried out at 15000 rpm for 10 minutes at the room temperature (CN-1050, AS ONE, Japan). Then, the supernatant solutions were prepared as the samples for SPR measurement. $PM_{1.1}$ sample was prepared as well as SDS-PAGE sample. We determined the Cry j 1 concentration and the reactivity between Cry j 1 antigen and Cry j 1 antibody about Cry j 1 extracted from pollen of each site by using the surface plasmon resonance method (SPR method).

2. Analytical Procedure of SPR Method

We evaluated reactivity between Cry j 1 antigen and Cry j 1 monoclonal antibody by using Biacore J system (Biacore J system, GE Healthcare, Japan) based on the SPR and a sensor chip (CM 5, GE Healthcare, Japan). First, 100 μL of N-ethyl-N'-(3-dimethylaminopropyl)carbodiimidehydrochoride solution and 100 μL of N-hydroxysuccinimide solution were added to a micro tube, and it was shaken. The mixed solution was injected to an injection port of Biacore J system and flowed for 6 minutes to activate the sensor chip. Next, 10 μg/mL of Cry j 1 monoclonal antibody (clone 013, Seikagaku Biobusiness, Japan) solution (diluted by pH 5.0 sodium acetate solution) as a ligand solution was injected to an injection port and flowed for 6 minutes to immobilize the ligands to a sensor chip. Then, 1 M ethanolamine hydrochloride solution as a blocking reagent was injected to an injection port and flowed for 6 minutes. In the last, sample solutions (Materials and Experimental Methods D. 1) was injected to an injection port and flowed for 2 minutes. All flow rates were 30 μL/min, and HBS-EP (BR-1001-88, GE Healthcare, Japan) was used as a buffer solution. 10 mM glycine-HCl solution (pH 2.0) was used as a reclaimed solution of a sensor chip. Analysis of intermolecular interaction was carried out by using BIAevaluation (GE Healthcare, Japan). Cry j 1 concentrations were determined by calibration curve for standard Cry j 1 (concentration range : 0 μg/mL ~ 5 μg/mL).

RESULTS AND DISCUSSION

A. Verification of Existence Form of Respirable Allergen Particles by An Immunofluorescence Technique with A Fluorescence Microscope

Fluorescence micrographs of the quartz fiber filters by an immunofluorescence antibody method showed in **Fig. 4**.

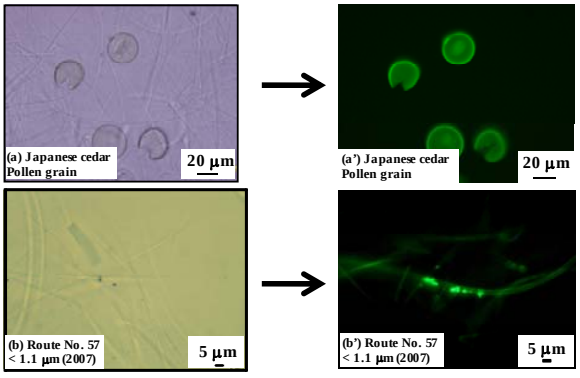

Fig. 4 Fluorescence micrographs of the quarts fiber filters by an immunofluorescence antibody method. ((a'): fluorescence of pollen grains, (b'): fluorescence of air sample (>1.1 μm), left micrographs were taken before B excitation light was irradiated).

The luminescent spots of green fluorescence luminescence were the airborne particles containing Cry j 1. As a result, it clarified that Cry j 1 exist below 1.1 μm and aggregate with the other airborne particles in the urban atmosphere. Therefore, we could verify the hypothesis that the aggregates containing Cry j 1 as an airborne microparticle (< 1.1 μm) may cause the Japanese cedar pollinosis and asthma by invading into a lower respiratory tract. Moreover, it was suggested that Cry j 1 may denature to another matter increased allergy response by aggregating with the other airborne microparticles.

B. Evaluation of Japanese Cedar Pollen Allergen Modification by Simulated Air Pollutants

1. SDS-PAGE profile of Japanese Cedar pollen extracts exposed pollutants

After Japanese cedar pollen extract (JCPE) were added to various pollutants, they were separated by SDS-PAGE and detected by silver staining method. The result showed in Fig. 5.

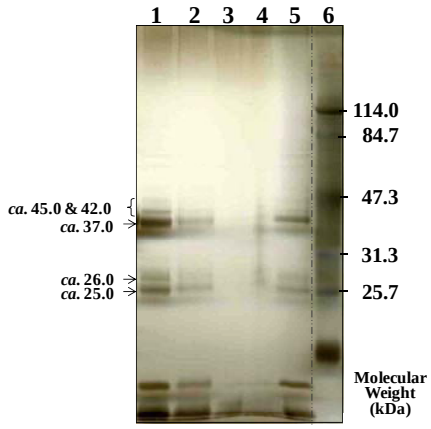

Fig. 5 SDS-PAGE profile using silver staining method.
Line 1: Japanese cedar pollen extract (JCPE), Line 2: JCPE + Peroxynitrite *aq*, Line 3: JCPE + HNO_3 *aq*, Line 4: JCPE + H_2SO_4 *aq*, Line 5: JCPE + H_2O_2 *aq*, Line 6: Molecular weight marker.

The JCPE band added peroxynitrite aq (Line 2) and H_2O_2 aq (Line 5) respectively showed the lower concentration protein bands than JCPE band (Line 1). On the other hand, protein bands corresponding Cry j 1 and Cry j 2 were not detected in Line 3 (added HNO_3 *aq* to JCPE) and Line 4 (added H_2SO_4 *aq* to JCPE) respectively. As a result, it found that JCPE chemically denature by homogeneous reaction with air acidic pollutants. Therefore, it was suggested that Japanese cedar pollen allergen may denature chemically in the precipitation during the pollen dispersal period. Moreover, low molecular weight allergen protein that may be generated by reacting with pollutants have a possibility to cause adjuvant effect. Especially, nitrated protein cause immune reaction and show higher immunogenicity. Therefore, it is very important to investigate the chemical morphology and behavior of nitrated protein and nitrated Japanese cedar pollen allergen (nitrated Cry j 1) in the urban air-polluted atmosphere.

2. SDS-PAGE Profile of Extracts from Air Samples

Protein extracted from an air sample collected by the

Andersen high volume sampler was separated by SDS-PAGE and detected by silver staining method. The result showed in Fig. 6. Although we attempted to detect the protein bands by the western blotting (chromogenic substrate is a tetramethylbenzidine) which apply an antigen-antibody reaction for protein detection, protein bands were not detected. It is important to investigate the denaturation of Japanese cedar pollen allergen in actual environment, but we did not detect the Cry j 1 protein bands that are very low concentration in the urban atmosphere (route No. 463) on Mar. 12th, 2008. From now on, it needs to consider the enrichment method of protein and highly sensitive measurement method (Electron Spin Resonance method).

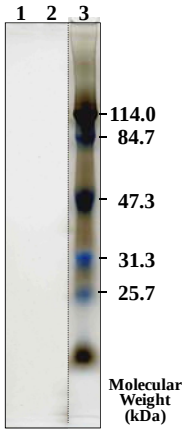

Fig. 6 SDS-PAGE profile using silver staining.
Line1: > 7.0 μm, Line 2: < 1.1 μm, Line 3: Molecular weight marker.
Air samples were collected at route No. 463 (Mar. 12th, 2008).

C. Evaluation of Intermolecular Interaction between Cry j 1 Antigen and Cry j 1 Antibody by SPR method

Table. 1 shows Cry j 1 concentrations and reactivity between Cry j 1 antigen and Cry j 1 antibody in the various sites based on the SPR method. As a result, it found that Cry j 1 concentrations in the pollens collected in various sites are almost constant (6.29 to 6.58 μg/ml), and the dissociation constants (K_D=K_d/K_a) calculated from coupling rate constants (K_a) and dissociation rate constants (K_d) were also almost constant (1.79 ~ 3.37×10^{-9} M). However, the constants of $PM_{1.1}$ containing Cry j 1 of urban area were different from these of the fresh pollen samples. Dissociation constant (K_D) of $PM_{1.1}$ is very lower (1.76×10^{-14} M) than that of the fresh pollen samples, which is assumed that the biomolecular interaction between Cry j 1 and Cry j 1 antibody become very strong and difficult to be dissociated. It support the hypothesis that Cry j 1 contained in the airborne fine particles (< 1.1 μm) is exposed to the urban polluted air and may be modified by the air pollutants during the transportation to the urban atmosphere.

If Japanese cedar pollen allergens (Cry j 1 and Cry j 2) may be modified by the air pollutants, it is necessary to examine its mutagenicity from now on. For example, nitrated polyaromatic hydrocarbons hava mutagenicity [13], and it cause negative health impact. Therefore, it is very important to confirm mutagenicity of modified pollen protein containing allergenic contents.

TABLE I
CRY J 1 CONCENTRATIONS OF VARIOUS SITES AND VALUES OF KA, KD AND KD BETWEEN CRY J 1 AND CRY J 1 MONOCLONAL ANTIBODY.

Samples (sites)	K_a (1/Ms)	K_d (1/s)	K_D (M)	Cry j 1concentrations (μg/mL)
Pollen (Saitama)	4.76×10^5	8.51×10^{-4}	1.79×10^{-9}	6.29 ± 0.231
Pollen (Ibaraki)	2.44×10^6	3.21×10^{-3}	1.32×10^{-9}	6.23 ± 0.256
Pollen (Tama)	1.76×10^5	5.92×10^{-4}	3.37×10^{-9}	6.58 ± 0.581
$PM_{1.1}$ (Saitama)	2.22×10^8	3.90×10^{-6}	1.76×10^{-14}	-

Here, coupling rate constants (K_a), dissociation rate constants (K_d) and dissociation constants (K_D), K_D=K_d/K_a

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AUTHOR'S BIOGRAPHY

Qingyue WANG was born in Shanghai, China, in December, 1959. This author graduated from Shanghai University (China) in 1982 with a master degree and earned the doctor degree from Saitama University (Japan) in 1995 in Environmental Science and Engineering. The major research subject and direction aim to development and application of solid waste treatment, utilization for energy and resources, coal cleaning and air pollution control techniques, also including research on determination of air pollutants including various pollen allergenic contents and source apportionment for environmental pollutants.

From 1995 to 2002, he worked as a SENIOR RESEARCHER and GENERAL MANAGER for Center for Research & Development of Environmental Conservation (C.E.C.) of IGNA Association, Japan; from 1996 to 1997, he worked as a GUEST RESEARCHER for Japan National Institute for Environmental Studies; from 1996 to 2001, he was employed as an ADVISER for Environmental Projects of the Yasuda Fire & Marine Insurance Co., Ltd, Japan; from 1995 to 2000, also as a LECTURER for Saitama University. From 2002, he is serving as ASSOCIATE PROFESSOR at Ecochemistry for Environmental Science, Department of Civil and Environmental Engineering in Saitama University, Japan. Up to now, he has many scientific publications, several papers of his recent research achievements are listed as follows:

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Prof. Wang has participated in Asian Journal of Atmospheric Environment as an Advisory Board from April, 2006 and became an Editorial Board of International Journal Editorial Committee in 2010. Prof. Wang has awarded as Japan Association of Aerosol Science and Technology in 1999, Japan Society for Atmospheric Environment in 2008 as well.