Release rate of daughter allergenic species from *Cryptomeria japonica* pollen grains trapped in air polluted wet deposition

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Abstract

High airborne concentrations of fine allergenic particles of *Cryptomeria japonica* pollen were determined during the pollen scattering spring seasons in Japan. In particular, this phenomenon was observed in field investigations during sunny days after rainfall. It is considered that the release of daughter allergenic species derived from pollen grains may be induced during urban rainfall events. Hence, it is necessary to do research in order to find the evidence of this phenomenon.

There are two major daughter allergenic species produced by pollen in Japan, which are called Cry j 1 and Cry j 2 of *Cryptomeria japonica* pollen. Allergenic Cry j 1 mainly exists in the pollen wall surface (Ubisch body), while allergenic Cry j 2 is present as a starch granule in the pollen grain, it can be thought that the release mechanism of allergenic Cry j 1 and Cry j 2 species in rainfall should be different. Since the release mechanisms are still not clear, therefore, the aim of this study is to clarify the important mechanisms of elution rate of two kinds of daughter allergenic species when pollen grains are washed out by rainfall and exposed to polluted air.

From the analytical results of rainwater samples and model laboratory experiments of simulated rainfall, it was indicated that a great number of pollen grains were trapped in initial rainfall. At the same time, many bursts of pollen grains were also observed in the rainwater. Thus, it is possible that the two daughter allergenic species will be released from the fractions of cell wall, burst

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pollen and the surfaces of pollen grains. Moreover, ionic concentrations in initial rainfall were higher than following wet deposition, because air pollutants were also abundantly trapped in the initial rainfall. Especially, it was found that the elution rate of allergenic Cry j 1 was increased when in contact with rainwater containing high ionic concentrations. In this case, elution of allergenic Cry j 1 was rapidly increased when in contact with a weak basic solution with high Ca^{2+} concentrations. Similarly, elution of allergenic Cry j 2 was also prompted from burst pollen washed out by rainfall and exposed to polluted air. Therefore, it is one important factor for the fast release of daughter allergenic species from pollen grains washed out by rainfall and exposed to polluted air including abundantly crustal particles such as road dust and Asian dust.
Keywords: Cryptomeria japonica pollen, allergenic Cry j 1, allergenic Cry j 2, air polluted precipitation, release rate, Asian dust, crustal particles.

1 Introduction

Japanese cedar pollinosis has been increasing in Japan and currently affects over 16% of the population (even up to 40% in the urban areas of Tokyo) (Nishihata *et al.* [1]). This prevalence is increasing in urban areas and it is currently about 28% (Bureau of Social Welfare and Public Health, Tokyo Metropolitan Government, Japan, [2]). According to our airborne sampling, *Cryptomeria japonica* pollen allergenic species distribute abundantly in the size range below 1.1 µm ($PM_{1.1}$) (Wang *et al.* [3]). It can be explained that these allergenic species are in the form of small size particles and can be released from coarse particles originated by *Cryptomeria japonica* pollen grains (about 30µm) to fine allergenic particles in $PM_{1.1}$. Therefore, these can be called daughter allergenic particles of *Cryptomeria japonica* pollen grains. *Cryptomeria japonica* pollen, so-called Japanese cedar pollen, has two main allergenic species, Cry j 1 and Cry j 2, which are responsible for pollinosis. Allergenic Cry j 1 exists in the pollen wall (Ubisch body), while allergenic Cry j 2 is present as a starch granule on the pollen grain (Nakamura *et al*. [4]). Daughter allergenic particles of *Cryptomeria japonica* pollen grains number increase during sunny days after rainfall (Wang *et al.* [5]). It is thought that daughter allergenic particles are responsible for inducing asthma by breaking into the lower respiratory tract. Actually, it is reported that symptoms of asthma patients were worsening during *Cryptomeria japonica* scattering season (Nakada *et al.* [6]). Especially, worsening of symptoms was notably observed at sunny days after rainfall (Taylor and Jonsson [7]). Therefore, we thought that rainfall was one important factor for the decrease in size of pollen allergenic species. Observations have reported that small-sized pollen allergenic species adhere to carbon aggregates from vehicle emissions (Namork *et al.* [8]). Since the worsening pollinosis symptoms are induced by aspiration when allergenic species coexist with air pollutants (Wang *et al.* [5]), known as adjuvants (Maejima *et al.* [9]), it is necessary to clarify the formation mechanism of the pollen allergenic species and air pollutants complex.

In solution, such as rainwater, elution of Cry j 1 is increased by solution with high ionic concentrations (Sagehashi *et al.* [10]). Because air pollutants are abundantly trapped into initial rainwater, ionic concentrations increased in initial rainwater. Moreover, ionic concentrations in rainwater changed by precipitation and air pollution conditions. In addition, there is the phenomenon of the long range transportation phenomena of Asian dust (AD) particles, which are transported from the East Asian continent into Japan by the westerly winds during the pollen scattering season in Japan, following global warming in recent years. When Asian dust is transported to Japan, pH and ionic concentrations in rainfall increased drastically (Kawamura and Hara [11]).

Nevertheless there is no research specifically focused on the release mechanism of allergenic species by various rainfall conditions until now. In this study, we investigated by two methods. The first aim of this study is to investigate the morphological behavior of *Cryptomeria japonica* pollen trapped in air polluted rainwater observed through rainfall sampling in an urban polluted area. And the second aim of this study is to clarify the elution rate through model laboratory experiments for understanding important elution mechanisms of allergenic species when pollen grains are washed out by rainfall and exposed to polluted air.

2 Materials and methods

2.1 Investigation of trapping counts and morphological behavior of *Cryptomeria japonica* pollen grains in urban polluted rainwater

2.1.1 Sampling procedure for rainwater in urban areas

Rainwater was collected in the urban area of Saitama city, located in the Kanto Plain, Japan. The mountainous forests of *Cryptomeria japonica* are around Chichibu city and neighbor prefectures far from the sampling site. Thus, *Cryptomeria japonica* pollen grains were transported from circumjacent mountainous areas, several hundred kilometers away. According to officially reported data from the Ministry of Environment, the scattering of AD in the Kanto region was observed with an annual average of three times, from 2001 to 2010, in pollen scattering spring season from February to April. Information of scattering of AD in the Kanto region was referenced from web site of the Ministry of Environment [12]. The days of observation of AD at the monitoring site at Tsukuba city was defined as the scattering days of AD at the sampling site.

Rainwater was collected with a rainwater sampler called Rain-go-round (Horiba, Co., Ltd.). This apparatus can partially collect rainwater from 1 to 7 mm of rainwater, collecting 5 mL per one mm rainwater from every event. This apparatus is equipped with a cap that automatically opens when rain starts, then, the penetration of dry deposition in collected rainwater was minimized. The sampling site was chosen carefully in order to block the influx of raindrops by the roof of buildings or trees. The sampling period was from early March till the end of April of 2010 during *Cryptomeria japonica* pollen scattering spring season. The collected rainwater samples were respectively set in micro tubes and centrifugally separated at 6,000 rpm for 10 min. Then, the supernatants were set in a polyethylene bottle. Supernatants and residues were stored at 4°C until analysis.

2.1.2 Measurement of trapping pollen counts and contents in rainwater

Burst of pollen grains in residue were counted separately from non-burst pollen grains by the following procedure; the residue was put on a glass slide, and then dried at 37°C overnight. Then, pollen grains were dyed by Phöbus Blackly distain solution prepared with 0.6 mg of methyl violet 2B, 30 mL of phenol, 180 mL of glycerin and 90 mL of ultra-pure water (Ariga [13]), and counted by using an optical microscope (Shimadzu Co., Ltd.) under 150 magnifications. Pollen on each slide was counted twice, and finally, average and standard deviation were calculated. Concentrations of ionic species and pH in rainwater were measured from the supernatants. The pH was measured with a pH meter B-212 (Horiba Co., Ltd.). Ionic concentrations of Cl^-, NO_3^-, SO_4^{2-}, Ca^{2+}, Na^+, Mg^+ and NH_4^+ were measured by an ion chromatography (DX-100, Dionex Co., Ltd.).

2.2 Laboratory elution experiments of *Cryptomeria japonica* pollen grains

2.2.1 Preparation for elution experiments

In order to investigate the elution mechanism of Cry j 1 and Cry j 2 from *Cryptomeria japonica* pollen grains, simulated rainwater samples were prepared by changing the pH and ionic concentrations in different solutions (table 1). Initial rainwater samples (IR) were used, because pollen grains were abundantly trapped into initial rainfall. Simulated rainwater samples (SR) were prepared based on the pH and ionic concentrations of rainwater samples determined during the field sampling period in Saitama, Japan. Initial rainwater samples when AD was observed (IR-AD) were also prepared, because pH and ionic concentrations in rainwater increased considerably due to trapped AD in it. These simulated rainwater samples were prepared in reference to our sampling result and data reported by other researchers (Kawamura and Hara [11] and Saitama Prefecture Official Website [14]).

Table 1: pH and ionic concentrations of simulated rainwater samples.

Rainwater samples*	pH	Ionic concentration (μM)									
		H^+	NH_4^+	Ca^{2+}	Mg^{2+}	K^+	Na^+	SO_4^{2-}	NO_3^-	Cl^-	CO_3^{2-}
IR	4.24	60	135	60	25	5	85	140	130	100	0
SR	4.85	20	30	10	0	0	10	40	30	20	0
IR-AD	7.85	0	200	300	50	10	100	250	210	200	50

*IR: initial rainwater samples; SR: simulated rainwater samples; and IR-AD: rainwater samples when AD was observed.

50 mg of pollen grain sample and 2 mL of each simulated rainwater sample or ultra-pure water were mixed in the micro tubes. Then, the micro tubes were stood at 4°C overnight. The supernatant in the micro tube was pipetted after centrifugal separation at 14,800 rpm by 10 min, and stored at -40°C until

analysis. Finally, allergenic concentrations in the supernatants were measured by Biacore J system.

2.2.2 Sample preparation and determination of elution rate of daughter allergenic species derived from *Cryptomeria japonica* pollen grains

The elution rate of Cry j 1 and Cry j 2 from pollen grain was measured in phosphate buffered saline (PBS) solution and in the simulated rainwater sample IR-AD, the experiments were performed by the following method: 200 mg of pollen sample and 1 mL of PBS or IR-AD were mixed in the micro tubes and stood in 4°C for 15, 60, 180, 360, 760 and 1440 min. After that time the micro tubes were centrifuged at 14,800 rpm by 10 min and the supernatants were pipetted. The supernatants were solvent-exchanged for HBE-EP solution (GE Healthcare Co., Ltd.) by the centrifugal filter units (Amicon Ultra – 0.5, Millipore Co., Ltd.). Solvent exchange was carried out according to the instructions of the centrifugal filter units. Finally, allergenic concentrations were measured by Biacore J system (GE Healthcare Co., Ltd.) based on the surface plasmon resonance (SPR) method.

2.3 Measurement of daughter allergenic species derived from *Cryptomeria japonica* pollen grains

Biacore J system based on the SPR method was used for the biochemical analyses, mainly used in immunological analysis to detect the presence of antibodies or its antigens in a sample. Cry j 1 and Cry j 2 concentrations were measured with a Biacore J system. This measuring method was adopted from the method reported by Takahashi *et al.* [15] with the improved procedure as follows: Allergenic Cry j 1 or Cry j 2 concentrations in measured samples were investigated based on Cry j 1 monoclonal antibody (MAb) or Cry j 2 MAb immobilized on a CM5 sensor chip (GE Healthcare Co., Ltd.) by using the SPR method with the Biacore J system. Approximately 700 μg of Cry j 1 MAb or Cry j 2 MAb was immobilized on the chip. The regeneration and dissociation of antigen-antibody complex reaction on the CM5 sensor chip were performed by 70 μL of pH 2.0 glycine-HCl (GE Healthcare Co., Ltd.). The analysis was repeated three times (n=3) for each sample for better accuracy. Finally, the results were converted into the concentrations of the allergenic Cry j 1 and Cry j 2 eluted from 1.00 mg pollen grains (μg/mg) in mass.

3 Results and discussions

3.1 Counting number of trapped pollen grains in rainwater

Figure 1 shows the time variation of airborne pollens and precipitation during spring 2010. Airborne pollen grains counts decreased with rainfall events. There were also days when non-decreasing counts of airborne pollen grains during rainfall events were observed, on these days it rained at night time when pollen grains were less scattered. During the spring period, rainwater samples were

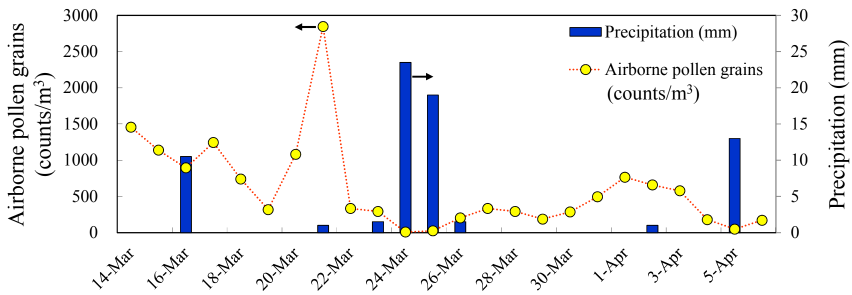

Figure 1: Time variation of airborne pollen counts and precipitation during the pollen scattering spring seasons.

Table 2: The variation of pollen counts collected in every 1.0 mm of rainwater after each event during the pollen scattering spring season.

Sampling data	Rainfall (mm)						
	1	2	3	4	5	6	7
16 Mar.	307 ± 31	29 ± 1	9 ± 0	2 ± 0	8 ± 0	3 ± 0	3 ± 0
21 Mar.	18 ± 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24 Mar.	34 ± 2	7 ± 0	n.d.	5 ± 0	3 ± 0	8 ± 0	6 ± 0
5 Apr.	271 ± 27	356 ± 46	101 ± 14	13 ± 0	10 ± 0	6 ± 0	2 ± 0

*Average ± standard deviation

collected 4 times on 16 March (10.5 mm), 21 March (only 1.0 mm), 24 March (24 mm) and 5 April (13 mm).

Table 2 indicates the variation of pollen counts collected in every 1.0 mm of rainwater after each rainfall event. Additionally, figure 2 indicates the collected ratio of pollen grains in every 1.0 mm of rainfall during each rainfall event,

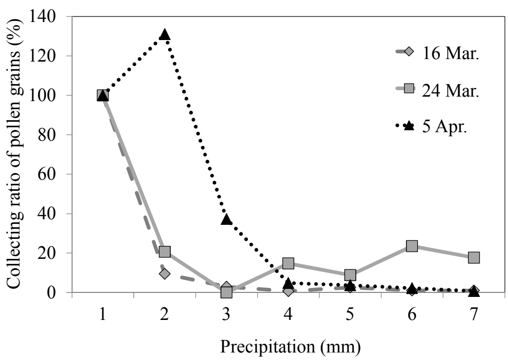

Figure 2: Collecting ratio of pollen grains in every 1.0 mm of rainfall during each rain event, normalized to the initial value (100%).

normalized to the initial value (100%), therefore, the 1.0 mm rainfall on 21 March, cannot be shown in figure 2. The highest standard deviation of pollen counts in rainwater was 14%. Most pollen grains were trapped into initial rainfall, with exception of 5 April when pollen grains trapped into the second 1.0 mm of rainfall rather than the first 1.0 mm. On this day, initial rainfall was light rain, 0.5 mm/h, then, airborne pollen grains could not be completely trapped into the initial rainfall. From this result, it was demonstrated that most *Cryptomeria japonica* pollen grains were trapped into initial rainfall. Other authors have also reported that *Cryptomeria japonica* pollen grains were trapped into initial rainfall (Saburi *et al.* [16]).

3.2 Ionic concentrations in different rain events

Ionic concentrations decreased with increasing precipitation (figure 3). Intermittent rain fell on 24 March and 5 April, air pollutants were scattered and trapped following rainfall. AD was observed on 20 March in Japan. Thus, AD was trapped into rainfall the next day, and ionic concentrations became higher than rainfall in other days. Especially, mineral ions such as Ca^{2+} derived from AD and/or road dust drastically increased about 2.5 times. From our previous report (Wang *et al.* [17]), elution concentration of Cry j 1 increased by solutions containing high ionic concentrations under weak basic conditions. Therefore, it suggested that elution of daughter allergenic species were induced by rainfall trapping of crustal particles (e.g. road dust and AD).

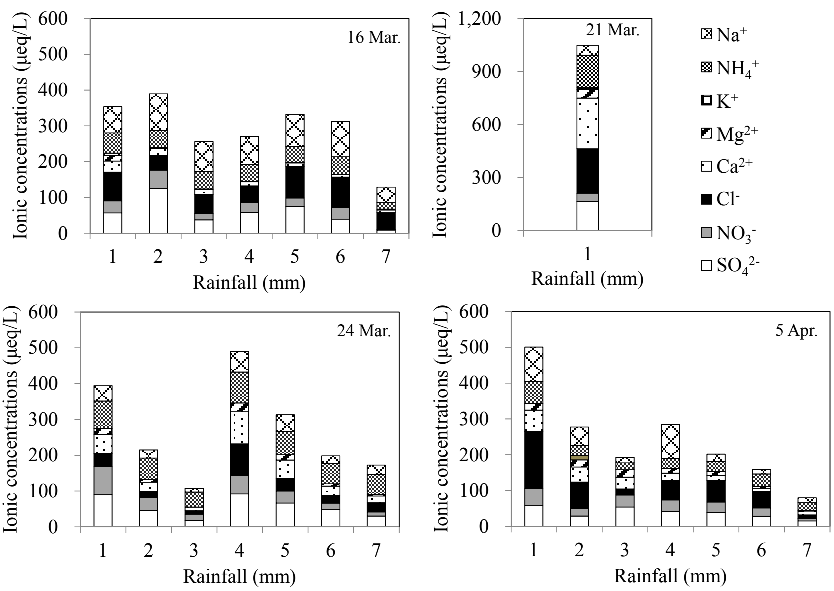

Figure 3: Ionic concentrations at different rain events.

3.3 Influence of pH on burst of pollen grains trapped into rainwater samples

Since elution of Cry j 2 was induced by a burst of pollen grains in contact with basic solutions (Nohara *et al.* [19]), we thought that elution of Cry j 2 was strongly depending on solution pH. Thus in this section, we investigated the burst ratio of pollen grains related with pH. Figure 4 indicates examples of morphological change of *Cryptomeria japonica* pollen grain, in which *Cryptomeria japonica* pollen burst in rainwater samples. Figure 5 indicates the correlation between pollen burst ratios and pH in rainwater. The data at 3rd mm of rainwater in 24 March was eliminated, because there were no pollen grains trapped in rainwater. Despite of a better correlation (R = 0.61, $p < 0.001$), the burst ratio of pollen grains was only 30% in weak basic rainwater. Therefore, we think that the burst of pollen grains might be also induced by other influence factors.

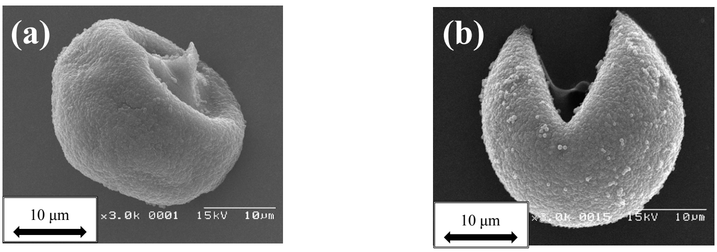

Figure 4: Examples of morphological behavior of *Cryptomeria japonica* pollen grain. (a) non-burst pollen grain and (b) burst pollen grain. (Pictures taken with a scanning electron microscope.)

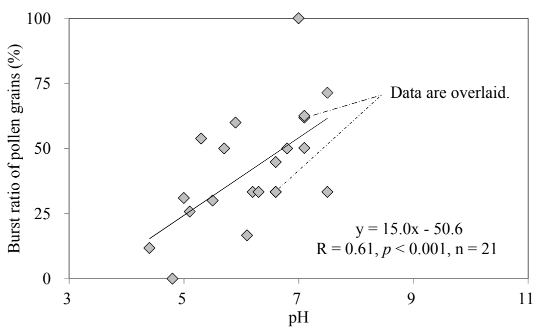

Figure 5: Correlation of pH and burst ratios of pollen grains in rainwater.

Scattering of AD was observed on 20 March, while our samples indicated that AD was trapped into rainfall on 21 March. Most pollen grains were burst in rainwater on 21 March. Rainfall trapping AD was reported to increase pH above 7.0 (Kawamura and Hara [11]), and pH in rainwater on 21 March also increased.

From this result, we thought that the burst ratio of pollen increased with increasing pH. Therefore, it is possible that rainfall trapping AD is one important factor that affects the release the pollen allergenic species of Cry j 1 and Cry j 2. Two major daughter allergenic species were released from pollen grain, and then suspended as fine particles containing allergens.

3.4 Elution behavior of daughter allergenic species derived from *ryptomeria japonica* pollens when contacted with simulated rainwater samples

Figure 6 indicates eluted Cry j 1 concentrations when in contact with various simulated rainwater samples. Elution of Cry j 1 was observed in IR-AD, IR, SR and ultra-pure water, in order to increase Cry j 1 elution concentrations. Based on the fact that the content of Cry j 1 in *Cryptomeria japonica* pollen is 435 ± 169 ng/mg (Fukuda *et al.* [20]), it was estimated that elution ratio of Cry j 1 was 3.8% in IR-AD, 0.6% in IR and 0.4% in SR respectively. From some reports of scattering counts of *Cryptomeria japonica* pollens in Japan (Wang *et al.* [3]), weight of pollen grain (Ohashi *et al.* [21]) and contained amount of Cry j 1 (Fukuda *et al.* [20]), it was estimated that Cry j 1 was eluted with 1.3, 0.9, 8.3 pg/m^3 by IR, SR and IR-AD respectively. Takahashi *et al.* [15] reported that symptom of pollinosis appeared when reaching about 1–3 pg/m^3 of *Cryptomeria japonica* pollen allergenic concentrations in the atmosphere. Therefore, worsening asthmatic symptoms may associate with the generation of small-sized allergenic species induced by rainfall and exposed to polluted air, especially AD.

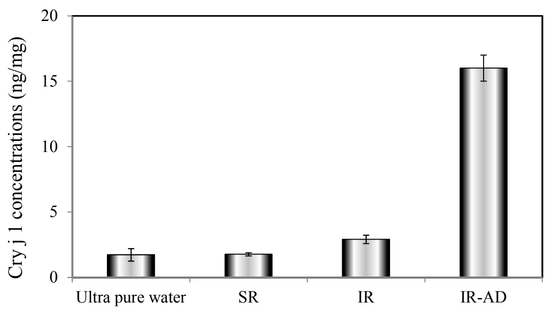

Figure 6: Eluted Cry j 1 concentrations when various simulated rainwater samples were in contact. IR: initial rain, SR: simulated rain and IR-AD: rain when AD is observed (n = 3).

3.5 Elution rate of daughter allergenic species derived from *Cryptomeria japonica* pollens

Eluted concentrations and variations through time of Cry j 1 and Cry j 2 were shown in figure 7. When pollen was in contact with PBS solution, most of the Cry j 1 was quickly eluted and almost kept stable. In addition, Cry j 2 elution was stable till 180 min. On the other hand, when pollen was in contact with IR-AD, Cry j 1 elution was also stable till 180 min. Nevertheless, few Cry j 2 eluted quickly, the eluted concentrations of Cry j 2 did not increase subsequently.

Elution of Cry j 1 was induced by solutions with high ionic concentration, despite the upward range of rainfall in the atmosphere (Sagehashi *et al.* [10]). Thus, it was thought that the elution rate of Cry j 1 was also influenced by ionic concentrations which were 150 mM and 0.66 mM, PBS and IR-AD, respectively. The differences of elution rate between Cry j 1 and Cry j 2 may be affected by localization of allergens. The elution rate of Cry j 1 was quicker because Cry j 1 was localized in the pollen wall surface in contrast to Cry j 2. Since the elution rates and concentrations of daughter allergenic species were induced by increasing pH and ionic concentrations in rainwater. Ionic concentrations in wet deposition that trapped pollen grains and deposited them on the ground were decreasing with increasing precipitation. Moreover, the elution of daughter allergenic species decreased gradually. Thus, we suggested that pH and ionic concentrations in initial rainfall were important factors affecting the elution rates of daughter allergenic species derived from *Cryptomeria japonica* pollens.

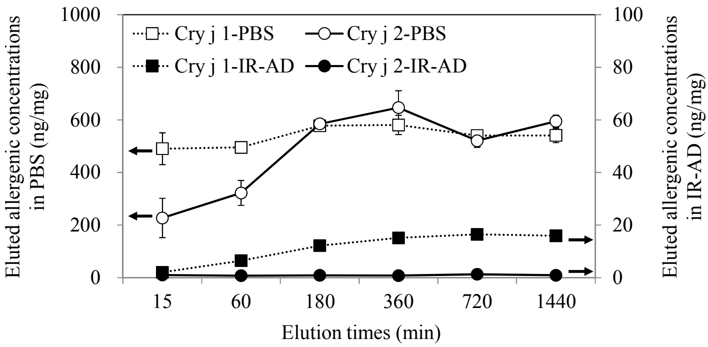

Figure 7: Eluted Cry j 1 and Cry j 2 concentrations (n = 3) and their time variations from 15 min to 1,440 min in PBS and IR-AD solutions.

4 Conclusion

In this study, we investigated the release behavior of daughter allergenic species from *Cryptomeria japonica* pollen grains trapped in rainfall and exposed to polluted air. From our results of rainfall sampling, most airborne pollen grains were trapped in initial rainwater. The ionic concentrations in initial rainfall were higher than the following one, because air pollutants were only abundantly trapped in initial rainfall. Most of the burst pollen grains were also observed in weak basic rainwater. Moreover, from the results of model laboratory experiments, different elution rates of allergenic Cry j 1 were observed in several simulated rainwater solutions. Especially, elution concentrations of Cry j 1 increased notably by IR-AD. Cry j 1 in IR-AD was almost eluted by 180 min. However, few Cry j 2 eluted quickly, elution of Cry j 2 did not increase subsequently. In conclusion, there may be two processes for release of *Cryptomeria japonica* pollen allergenic species related with air polluted wet deposition. One is the elution of allergenic species (mainly Cry j 1) from pollen grain surface by contact with rainwater, and the other is the release of allergenic species (both of Cry j 1 and Cry j 2) from burst pollen grains when trapped in rainwater. Based on the evidence obtained from this study, it can be thought that daughter allergenic species are easily released during rainfall, and then, re-suspended as allergenic fine particles in the urban atmosphere during sunny days after a rainfall event.

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