

# *Amaranthus* species around Bangkok, Thailand and the release of allergenic proteins from their pollens

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## Summary

**Background:** Pollen of *Amaranthus* L., commonly known as careless weed or Phak-khom in Thai, has become one of the major causes of airway allergy in many countries including Thailand. Despite its recognized importance, there is no available information about which *Amaranthus* species are producing allergenic pollen more likely to affect Thai patients. Furthermore, only allergenic proteins released from pollen can cause allergy.

**Objective:** This study aimed to survey species of *Amaranthus* found in Bangkok and to investigate the impact of water on pollen damage and protein release from *Amaranthus* pollens.

**Methods:** *Amaranthus* inflorescences were sampled and identified using the identification key provided in “Flora of Thailand”. Shed pollens were collected on day 1, 3 and 7 after shedding. Ten mg of pollens in distilled water including damaged pollens were counted under a light microscope. In addition, supernatant was analyzed for concentration of proteins released from pollens using Bradford’s assay. Profiles of released proteins and IgE binding proteins were analyzed by SDS-PAGE and Western blot.

**Results:** Three species of *Amaranthus*—*A. hybridus*, *A. spinosus*, and *A. viridis* were identified. SDS-PAGE analysis revealed at least twelve protein bands with MW ranging from 10 to 80 kDa. Water caused more damage to pollens and higher amount of proteins were recovered from pollens 1 day after shedding than from 3- and 7-days old pollens. The results of Western blot showed IgE-bound proteins with MW ranging from 30 to 50 kDa.

**Conclusion:** Water could damage pollens and time after shedding and significantly affected the amount of allergenic proteins released from pollen. (*Asian Pac J Allergy Immunol* 2015;33:203-10)

**Keywords:** allergy, *Amaranthus*, Bangkok, pollen, protein, water

## Introduction

The prevalence of allergy in Thais has been increasing in the past decade<sup>1</sup> and pollens have become one of the major airborne allergens in Thailand. However, information relating to pollens of local plants causing allergy remain insufficient. Although it is widely known that pollen allergy is often a seasonal disease affecting individuals when a particular type of flower is in bloom,<sup>2,3</sup> it is difficult to pinpoint the exact source of pollen unless a field survey is conducted because the blooming period is less markedly defined in tropical regions. An airborne pollen survey in Thailand showed that *Amaranthus* pollen was among the most commonly found pollen types. Moreover, Thai allergic rhinitis patients were shown to have sensitivity to an extract from *Amaranthus* pollen.<sup>4</sup>

*Amaranthus* L. species are abundantly distributed along road sides and waste areas. Six species of *Amaranthus* have been identified in Thailand, *A. spinosus* L., *A. caudatus* L., *A. hybridus* L., *A. tricolor* L., *A. viridis* L., and *A. lividus* L.<sup>5</sup> These plants produce large amount of pollens that are often detected in airborne allergen surveys.<sup>6,7</sup> Different *Amaranth* species had similar characteristics and can be easily mistaken.

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Amaranth inflorescences are borne in spikes or plumes and white, green, pink, or purplish. Pollen grains are yellow, spheroidal and polyaperturate (golf ball-like) with 30-65 apertures.<sup>8</sup>

Until now, there is no information available about which *Amaranthus* can be found around Bangkok region, where many allergic patients reside. Therefore, morphological descriptions of *Amaranthus* are necessary to accurately identify *Amaranthus* species. Species identification is needed because different species may produce pollens with different allergenic components. Furthermore, clinicians and patients may find this information useful in order to recognize and avoid these allergenic species. This study, therefore, aimed to survey *Amaranthus* species that most likely produce allergenic pollens around Bangkok and describe the morphology of each species. The extracts from pollen of these species were compared.

Most pollen allergic patients present IgE against specific protein components contained within the cytoplasm of pollen. Unless these proteins are released from the pollen, they cannot react with the immune system and cause allergic reactions.<sup>9</sup> Naturally, pollens undergo a process of dehydration before being released from anthers and will be rehydrated slowly by the stigma secretion in order to germinate and fertilize the female gametes. However, pollens can also break in other circumstances.<sup>10</sup> In the case of allergenic pollens, pollen wall is easily ruptured upon contact with water, tear, oral, nasal, or conjunctiva mucosa, causing the release of allergenic components that can quickly elicit allergic symptoms.<sup>9,11,12</sup> Moreover, allergenic components released under high humidity can also cause allergy as airborne particles.<sup>9,11,13,14</sup> This study investigated the dynamics of pollen damage and allergenic components released from *Amaranthus* pollens upon contact with water.

## Methods

### *Plant identification and pollen collection*

*Amaranthus* inflorescences were collected from sites within Bangkok metropolitan region. Plant voucher specimens were stored at the Department of Plant Science, Faculty of Science, Mahidol University. Samples were dissected under a stereomicroscope and identified to species using the identification key from Flora of Thailand.<sup>5</sup> Freshly cut inflorescences were arranged on flora foam soaked in water. Naturally shed pollens in a closed room were collected at day 1, 3 and 7 after

shedding. After separation of extraneous materials, pollens were passed through sieves to obtain >95% pure pollens.

### *Pollen crude extract preparation*

*Amaranthus* pollens were ground in liquid nitrogen and extracted in PBS buffer containing 1 mM PMSF. Protein concentration was determined using Bradford's assay. Crude extracts were separated with SDS-PAGE and stained with Coomassie Blue R-250 (Merck, Germany).

### *SDS-PAGE*

14% separating gel and 7% stacking gel were used. Samples were mixed with sample buffer and heated at 95°C for 5 minutes before being loaded into the gel to irreversibly denature protein. Proteins were mobilized with 28 mA per gel for 60-90 minutes.

### *Preparation of released proteins from pollen*

Ten milligrams of pollen was suspended in 1 ml solution and mixed gently. Then, 1 µl of the suspension was immediately applied onto a glass slide to count damaged pollen grains. The supernatant was also immediately collected (T<sub>0</sub> time point). The precipitated pollen was resuspended with fresh solution, mixed gently and incubated for 15 minutes at room temperature. Then, 1 µl of pollen suspension was investigated for damaged pollen grains (T<sub>0-15</sub>) and the supernatant was immediately collected. The procedure was repeated to prepare T<sub>15-30</sub>, T<sub>30-45</sub>, T<sub>45-60</sub>, T<sub>60-120</sub>, and T<sub>120-overnight</sub> in the same manner.

### *Investigation of pollen damage percentage*

Normal and damaged pollen grains were counted under the light microscope. Pollen suspension (1 µl) was immediately applied onto a glass slide, using Calberla's solution as mounting media. Percentage of damaged pollen was calculated from following equation.

### *Investigation of released proteins from pollen*

Pollen supernatant was collected at six time points T<sub>0</sub>, T<sub>0-15</sub>, T<sub>15-30</sub>, T<sub>30-45</sub>, T<sub>45-60</sub>, T<sub>60-120</sub>, and T<sub>120-overnight</sub>. Protein concentration was determined using Bradford's assay according to the manufacturer's recommendation (Bio-Rad, USA). Quantification of protein was performed by comparison with the standard curve constructed using BSA. Protein profile was investigated by SDS-PAGE. Released proteins were separated and stained with Pierce® silver stain (Thermo scientific, USA) according to the manufacturer's recommendation.

### Serum sample

Sera from three atopic donors with positive SPT result ( $\geq 3$  mm) using *Amaranthus* pollen extract and sera from three normal donors were obtained in collaboration with Development of Siriraj pollen Allergen Vaccine (SPAV) project, Faculty of Medicine, Siriraj Hospital (SiEc100/2012).

### Immunoblot analysis

Separated protein bands of *Amaranthus* pollen extract were electro-transferred to nitrocellulose membranes at a constant 200 mA at 4°C for 2 hours in Semi-dry blotting apparatus (Cleaver Scientific, United Kingdom). After 2 hours, the membrane was stained with 0.1% Ponceau solution to confirm the transfer. The membrane was washed several times with distilled water and with washing buffer (PBS containing 0.2% Tween20) during the last wash, incubated in blocking solution (PBS containing 3% skim milk) for 1 hour at room temperature and then incubated with 1:16 diluted serum at 4°C overnight. After washing off the serum, the membrane was incubated with 1:10,000 dilution of HRP-labeled anti-human IgE antibody at room temperature for 1 hour and incubated with chemiluminescence HRP substrate for 5 minutes. Emitted signal was detected by x-ray film.

### Statistical analysis

An ANOVA was performed to evaluate the significance of the variation of damaged pollen percentage and released protein concentration among the different groups under investigation. Tukey post hoc tests were also used to assess differences among levels of treatments. All statistical analyses were performed using SPSS V.16.

## Results

### *Amaranthus* characteristics

Three species of *Amaranthus* were identified—*A. hybridus* L., *A. spinosus* L. and *A. viridis* L. All were annual erect herb with spirally arranged leaves. *A. hybridus* was the tallest species (up to 1.5 m) and *A. viridis* was found to be noticeably shorter (approximately 80 cm or less) than the other two species. These three species had leaf shape inflorescences, fruits, and seeds also exhibiting different characters, which could be used to identify *Amaranthus* to the species. *Amaranthus spinosus* and *A. hybridus* had ovate leaf shape, 5 perianths, 5 stamens and circumscissile fruits. Importantly, *A. spinosus* presents a special character, 2 spines at the

leaf axils. On the other hand, 3 perianths, 3 stamens and indehiscent fruits were found in *A. viridis* (Table 1, Figure 1). Pollens of these three species had similar characteristics. They were 13-36 micrometers in diameter with yellow color. Under the light microscope, pollen grains were monad, spheroidal, and polyaperturate (Figure 1-D). Of these three species, *A. hybridus* and *A. viridis* were found more abundantly than *A. spinosus*, which was mostly found in agricultural areas.

SDS-PAGE analysis showed similar protein profiles from crude extracts of *A. hybridus*, *A. spinosus*, and *A. viridis* (Figure 2). Each crude extract contained at least twelve bands with estimated molecular weights ranging between 10-80 kDa, including 10, 15, 22, 25, 33, 35, 40, 43, 50, 55, 60, and 72 kDa. Relative concentrations of each protein band were similar in all extracts.

### Investigation of pollen damage upon contact with water










Water impact on pollen damage and protein release was investigated using naturally shed pollen from *Amaranthus hybridus* as a representative species. Pollen was incubated in distilled water for 0, 15, 30, 45, 60, 120 minutes and overnight. Damaged and undamaged pollen grains were counted under light microscope (Figure 1-D). The results showed that the number of damaged pollen grains increased as the incubating time increased. At  $T_0$  time point, the percentage of damaged pollen was about 22.94%. Pollen was damaged up to 50% at  $T_{45-60}$  time point. The highest percentage of visibly damaged pollen was approximately 60.26%, even after an overnight incubation (Figure 3-A).

To investigate whether pollens age can influence water-induced damage influence on, pollens collected after 1, 3 and 7 day of shedding were used. The percentage of damaged pollen 3 days and 7 days after shedding was found to be significantly lower ( $p$ -value  $< 0.05$ ) than 1 day after shedding at each time point. Pollen 3 days and 7 days after shedding were approximately 30% and 50% less damaged compared to pollen 1 day after shedding (Figure 3-C).

### Investigation of released proteins and allergens from damaged pollen

Most allergenic proteins are found in the pollen cytoplasm and must be released from pollen to cause allergic symptoms.<sup>15</sup> Released proteins from Amaranth pollen were analyzed for concentration and size. Bradford's assay showed that the highest

**Table 1.** Comparison of morphological characters of three *Amaranthus* species

Character \ Species	<i>A. hybridus</i>	<i>A. spinosus</i>	<i>A. viridis</i>
Height	1-1.5 m	1 m	30-80 cm
Leaf arrangement	Spirally without spine	Spirally with 2 spines	Spirally without spine
Leaf shape	Ovate 	Lanceolate 	Ovate 
Inflorescence	erect	erect	erect
Flower	Unisexual, green or reddish	Unisexual, green	Unisexual, red
Perianth	5	5	3
Stamen	5	5	3
Stigma	3	3	3
Fruit	Circumscissile 	Circumscissile 	Indehiscent 
Seed color	Brown or black,	Brown,	Dark brown,
Seed shape	Lenticular 	Orbicular 	Lenticular 

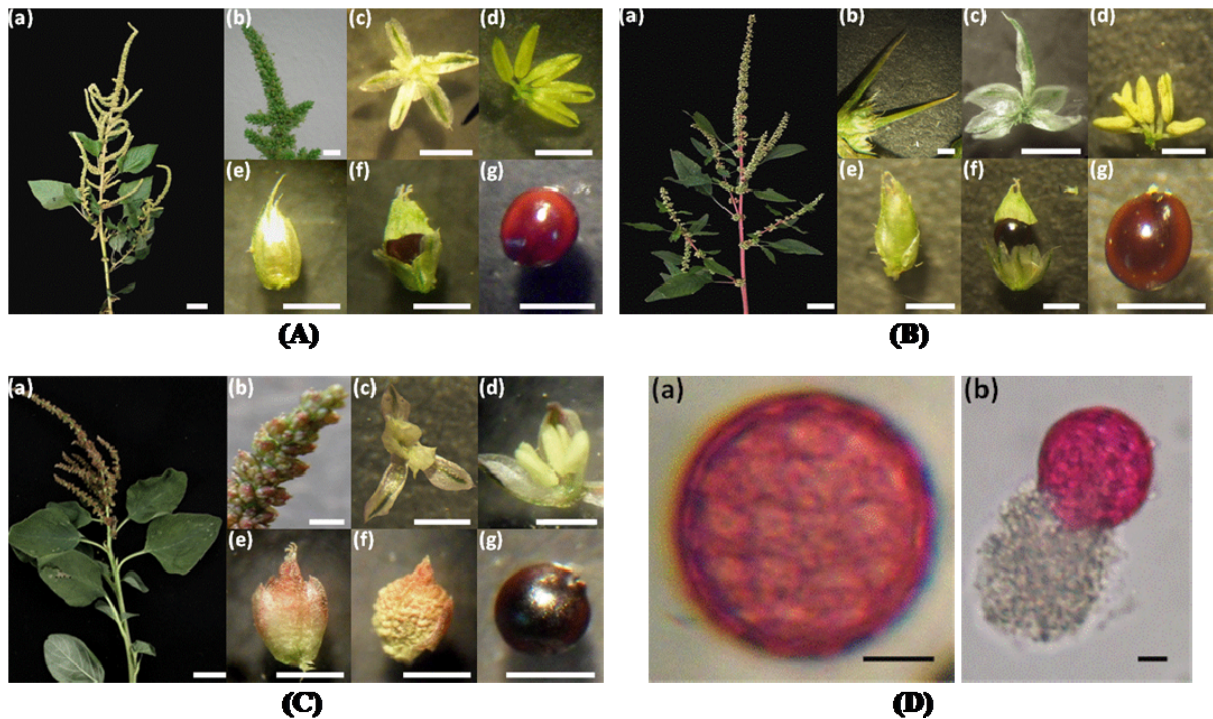
concentration of proteins was released from Amaranth pollen within the first 15 minutes after incubation. In fact, as much as 200 µg/ml of proteins was released almost immediately ( $T_0$  time point). The amount of released proteins steadily decreased after 15 minutes (Figure 3-B). Concentrations of proteins released from pollen 1 day, 3 days, and 7 days after shedding were significantly different at  $p$ -value  $< 0.05$ . Seven-day old pollen released only about 30% of the amount of proteins released by fresh pollen (1 day after shedding) (Figure 3-D).

Pattern of released proteins after incubation in distilled water was analyzed using SDS-PAGE and silver staining. The results showed that two prominent protein components with MW 40-50 kDa were released into solution (Figure 4). When comparing proteins released from pollens and total

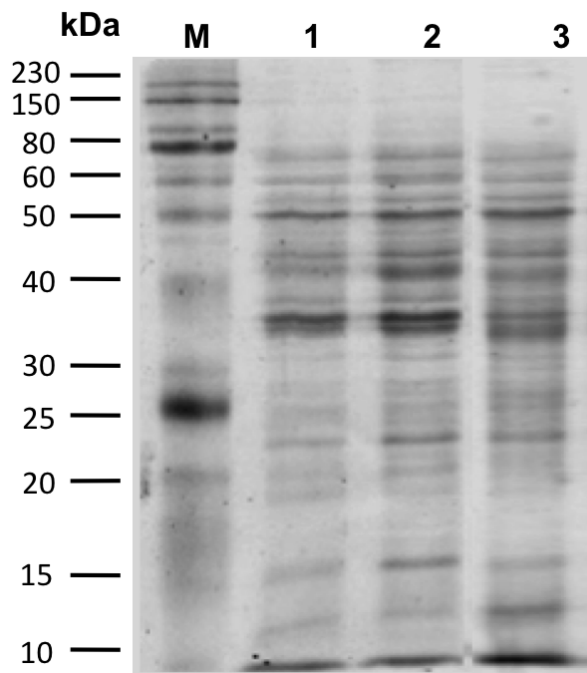
proteins extracted by grinding, total protein extract contained more prominent components. In accordance with the percentage of damaged pollen, proteins were mostly released from pollen within the first 15 minutes after contact with distilled water. Even though proteins released by pollen 7 days after shedding had lower band intensity, the protein pattern was similar to released proteins from 1-day old pollen.

To determine which allergenic components were released by Amaranth pollen upon contact with water, pooled serum of three Amaranth SPT-positive donors was used. The results showed specific IgE bound to allergens with MW ranging from 30 to 50 kDa (Figure 5). Moreover, all allergens were released within the first 15 min from damaged pollens of both day 1 and 7 after shedding





**Figure 1.** *A. hybridus* (A), *A. spinosus* (B), and *A. viridis* (C) plant characteristics: (a-b) Inflorescence erect; (c) Perianth; (d) Stamen; (e) Female flower; (f) Fruit; (g) Seed (Scale bar (a) = 1 cm, (b-g) = 1 mm). *Amaranthus* pollen (D): (a) *Amaranthus* pollen; (b) damaged pollen (Scale bar = 10  $\mu$ m)



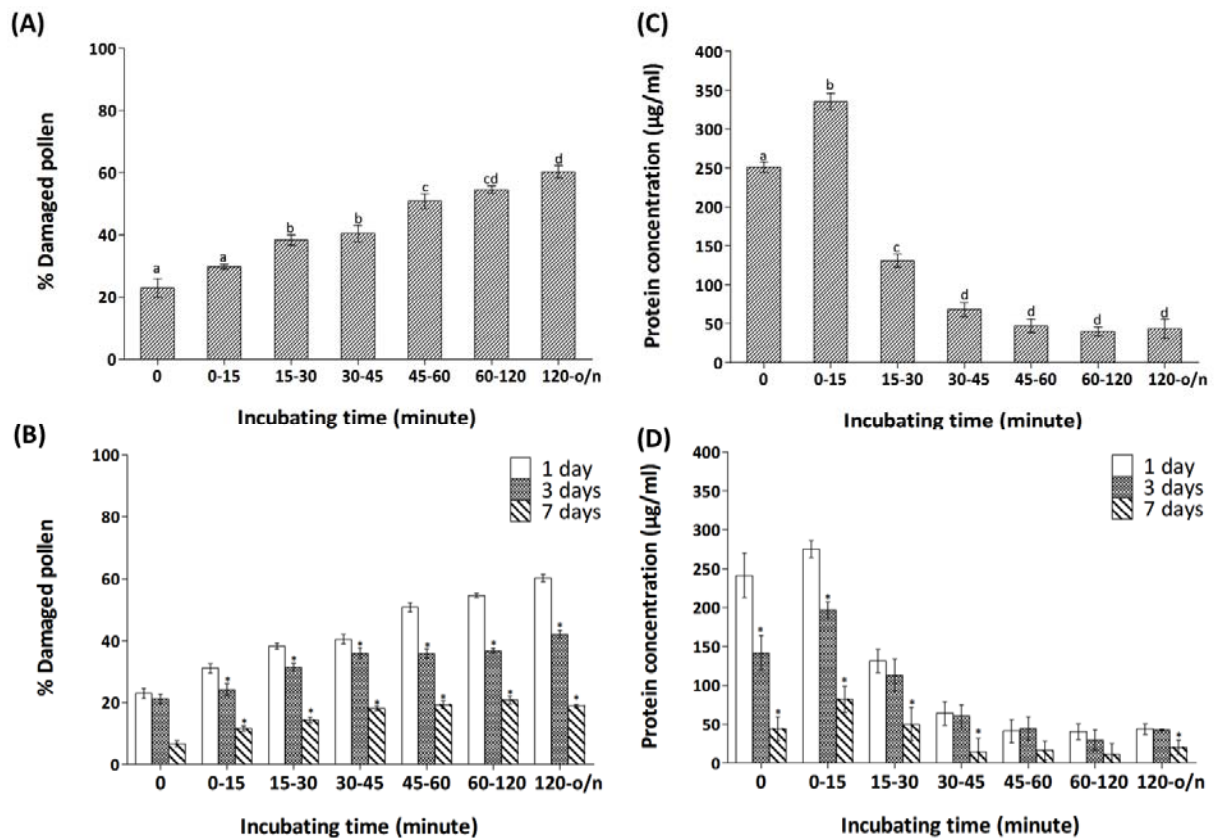
**Figure 2.** SDS-PAGE analysis of total protein extract of three pollens from *A. hybridus* (Lane 1), *A. spinosus* (Lane 2), and *A. viridis* (Lane 3) on 14% SDS-PAGE gel stained with Coomassie Brilliant Blue. Note: Lane M = Protein Marker (NEB<sup>®</sup>, England)

(Figure 5-A, B). Interestingly, serum specific IgE also bound to ~26 kDa allergen only in total protein extract (Figure 5).

### Discussion

Pollen allergen surveys showed that pollen of *Amaranthus* can be found in the atmosphere throughout the year.<sup>6,15</sup> A study performed in Spain showed that the maximum concentration of *Amaranthus* pollen was found between June and October each year<sup>6,15,16</sup>, while another study performed in Romania showed the highest daily pollen concentration between late July and mid-August<sup>7</sup>. Previous airborne pollen survey in Bangkok showed that the peak of *Amaranthus* pollen count occurred between November and January with the highest concentration in December.<sup>17</sup> Climate factors affecting pollen abundance are temperature, humidity, wind speed and rainfall but only temperature was found to have a significant effect on *Amaranthus* sp. pollen concentration.<sup>6,15</sup>

Although six species of *Amaranthus* can be found in Thailand, this study identified only three species: *A. spinosus*, *A. hybridus*, and *A. viridis*,



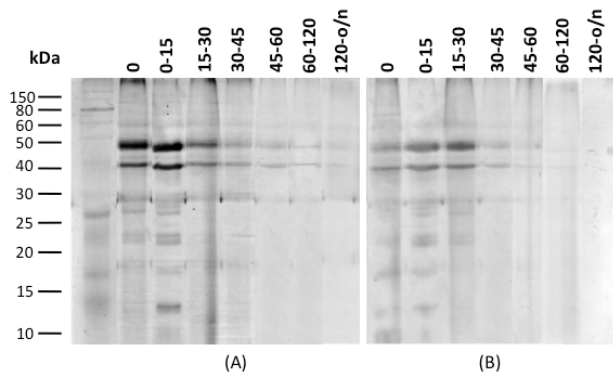
**Figure 3.** Comparison of damaged pollen percentage (A, B) and released protein from pollen (C, D) at each time point (A, C) and Time after shedding (B, D). Damaged pollen grains were counted under a microscope. Protein concentration of released proteins from damaged pollens was measured using Bradford's assay. Each value represents the mean of three independent experiments with standard error of mean (SEM). \*  $P$ -value < 0.05 compared with one another at each time point by one-way ANOVA and the Tukey post hoc test.

commonly found in the Bangkok Metropolitan Region. These three species had different characteristics. *Amaranthus spinosus* was easily distinguished by the presence of spines. The other two species could be identified by inflorescence size, number of perianths and fruit type. *A. hybridus* and *A. viridis* were distributed more abundantly than *A. spinosus*. Importantly, *A. hybridus* could produce larger and more branched inflorescences than the others, so a higher amount of pollens could be released per plant per day. On the other hand, *A. viridis* had small inflorescences and therefore was assumed to not contribute significantly to airborne pollen allergy in Bangkok.

The SDS-PAGE analysis of proteins from *A. hybridus*, *A. spinosus*, and *A. viridis* pollens showed very similar profiles. Total proteins were separated into twelve protein bands with molecular weights ranging between 10-80 kDa. One previous study showed 22 protein bands of *A. spinosus* pollen crude extract with molecular weights of 17-70 kDa.<sup>18</sup> The

observed variation in protein profiles could be due to variation in environmental factors and/or sensitivity of the analysis.<sup>19,20</sup>

Natural pollen is rehydrated immediately upon contact with wet surface, which can lead to pollen wall rupture and protein release from pollen.<sup>10</sup> Grass pollen allergens had been shown to localize in the cytoplasm and were released when pollen grains ruptured in water.<sup>12,21,22</sup> There are several factors affecting protein released from pollen incubated in solutions such as water, rain, high humidity condition, or/and air pollution.<sup>9,11,13,14</sup> This study demonstrated that incubating time and time after shedding significantly impacted Amaranth pollen damage and protein release. Immediately after contact with distilled water, about 20% of Amaranth pollen was damaged and released about 200 µg/ml of proteins per 10 mg pollen grains. As incubating time increased, the percentage of damaged pollen also steadily increased, but never more than 60%. Seven days old pollen was 70% less damaged than

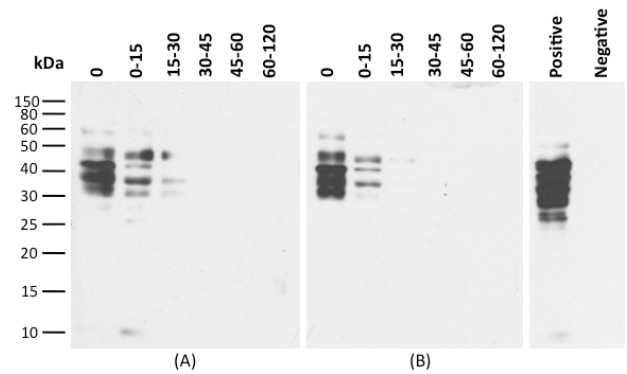


**Figure 4.** SDS-PAGE gel analysis of released protein from pollens. The released proteins from damaged pollens after incubated in water at indicated time were separated in SDS-PAGE gel and stained with silver stain. Note: A = pollens at day 1 after shedding, B = pollens at day 7 after shedding.

pollen 1 day after shedding. Similarly, the amount of released proteins from 7 days old pollen was only about 30% compared to proteins released from pollen 1 day after shedding.

To date, allergenic proteins of three species of *Amaranthus*; *Amaranthus retroflexus*<sup>23</sup>, *A. spinosus*<sup>24</sup> and *A. viridis* (unpublished data), have been studied. However, only one allergenic component of *Amaranthus* pollen, Ama r 2, from *A. retroflexus* had been identified and sequenced. Ama r 2 is a 14.6 kDa profilin protein composed of 133 amino acids.<sup>23,25</sup> The present study is the first report showing specific serum IgE from Thai Amaranth allergic donors bound to 4 different released novel allergens ranging 30-50 kDa and a novel ~26 kDa allergen in total protein extract. Profilin did not seem to be a significant allergenic component among Thai patients. Further identification of allergens as well as determination of major allergens are in progress.

Interestingly, despite 20% of damaged pollens in the first 15 minutes, it appeared that all allergens were released in the first 15 minutes as shown in the results of IgE immunoblotting. Although, only 23% of tested Amaranth pollens were also ruptured by the first 15 min in water, released allergens were easily detected suggesting that released allergens of Amaranth pollens could be one of the major causes of seasonal airborne allergy. Noteworthy, Amaranth



**Figure 5.** Immunoblotting analysis of released allergens from pollens. Specific IgE (pooled serum of 3 Amaranth-SPT positive donors) bound released proteins from damaged pollens after incubated in water at indicated time. Note: A = pollens at day 1 after shedding, B = pollens at day 7 after shedding, Positive = total protein extract, Negative = total protein extract incubated with pooled serum of 3 normal donors.

pollens of 1 day after shedding would induce greater allergic reactions than aged Amaranth pollens corroborating other studies<sup>21</sup> that found that fresh pollen is likely to induce more severe symptoms than aged pollens in sensitized patients.

In conclusion, this study found that *A. hybridus*, *A. spinosus*, and *A. viridis* were three species of the *Amaranthus* genus found in Bangkok Metropolitan Region. *A. hybridus* is likely to contribute most significantly to the Amaranth airborne pollen. Protein profiles of these three species were similar. The total protein extract contained nine major bands ranging between 10-80 kDa, while released proteins from damaged pollens showed two prominent components with 40 and 50 kDa. Furthermore, water could damage pollens and time after shedding significantly affected the amount of allergenic proteins released from *A. hybridus* pollen. Finally, there were 5 novel allergens released from damaged pollens and found in total protein extract.

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### Conflict of interest

All authors have no conflict of interest regarding this paper.

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