



Rapid pollen rupture and release of pollen cytoplasmic granules upon hydration of allergenic grass and weed species commonly found in subtropical regions

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Abstract Airborne pollen is an important and potent source of allergens. Allergenic protein components located in the cytoplasm of pollen, so-called pollen cytoplasmic granules, are released upon contact with moisture, thereby inducing allergic symptoms. However, few studies have compared factors affecting pollen rupture and protein release from allergenic pollen. This study investigated the dynamics of pollen rupture and protein release upon hydration using common allergenic grass and weed species in subtropical regions. The three factors examined were incubation time, incubating solution, and pollen age after shedding. We found that pollen rupture and protein release occurred rapidly upon contact with solutions. The highest amount of protein was released within 15 min after incubation. As expected, pollen rupture increased as incubation time increased. Fresh pollen had higher rupture percentage and released a higher amount of protein than aged pollen. Rainwater

caused more rupture and triggered more protein release than other tested solutions. The dynamics of rupture and the amount of released protein were drastically different among species. In conclusion, incubation time, incubating solution, and age of pollen significantly affected the amount of pollen rupture and protein release from allergenic pollen. This information provided better understanding of factors involving the release of allergenic protein from pollen in relation to its internal and external conditions, which could affect its allergenicity.

Keywords Allergen · Released protein · Ruptured pollen · Pollen cytoplasmic granules · Pollen allergy

1 Introduction

Airborne pollen is an abundant and potent source of airborne allergens. Concentration of airborne pollen and released allergens can help predict allergen dose and improve management of allergy and asthma attacks (Taylor et al. 2002; D'Amato et al. 2016). However, the amount of airborne pollen alone may not correlate well with allergy symptoms. Pollen allergenicity is partly due to the combined immunoreactivity of individual allergenic components, which has been studied extensively in the past decades, and bioavailability of allergenic components, a factor that

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has not yet been thoroughly explored (Radauer and Breiteneder 2006).

Grass and tree pollen is among the most important aeroallergens worldwide. Sensitization to pollen is strongly associated with allergic symptoms, especially in patients with allergic rhinitis (Pongsiripipat et al. 2009). There is significant prevalence of pollen sensitization in allergic patients living in tropical countries. Based on skin prick test reactions among Thai allergic rhinitis (AR) patients, Bermuda grass (52.3%), para grass (49.4%), sedge (45.9%), and careless weed (45.4%) were important causes for allergic rhinitis sensitization (Bunnag et al. 2009). Moreover, sIgE reactivity to Bermuda grass, Johnson grass, and para grass was found in about 70% of allergic rhinitis patients (Pacharn et al. 2019).

During development, pollen components, including allergenic proteins, are encapsulated in the cytoplasm or embedded in the pollen wall (intine and exine layers). Unless these components are released from the pollen, they cannot react with the immune system and cause allergic reactions (D'Amato et al. 2007). Mature pollen undergoes a process of dehydration before being released from anthers and becomes rehydrated by stigma secretion in order to germinate and fertilize female gametes (Firon et al. 2012). It is critical that the natural rehydration process occurs in a well-controlled manner, without causing cell damage or spilling cell contents. However, if pollen grains come into contact with a hypotonic solution such as rainwater or tears, water is rapidly absorbed into pollen grains and causes pollen wall rupture (Firon et al. 2012; Grote et al. 2000). The rupture releases cellular contents in small particles called pollen cytoplasmic granules (PCGs), containing allergenic proteins, enzymes, and bioactive lipid mediators that might be involved in triggering allergic symptoms (Behrendt and Becker 2001). Allergenic proteins in PCGs have been shown to elicit allergic symptoms upon contact with water, tears, and mucosa from the mouth, nose, or eyes (Behrendt and Becker 2001; D'Amato et al. 2007; Songnuan 2013).

For wind-pollinated plants, moisture can rupture pollen that has been dispersed or still remains in the anther. These pollen fragments can be released as a respirable aerosol. Airborne particles of allergenic components released under high humidity can stay airborne over a long period and are usually not accounted for in a regular airborne pollen survey. Importantly, they can penetrate deeper into the

airways, triggering allergic and asthmatic symptoms (Breiteneder 2008; D'Amato et al. 2007; Grote et al. 2001). It is believed that this phenomenon is the cause of thunderstorm-related asthma that can result in severe consequences and mortality in pollinosis patients (D'Amato et al. 2016). In 2017, thunderstorm-related asthma attacks sent thousands of patients to an emergency room and resulted in a few fatal cases in Melbourne, Australia (D'Amato et al. 2017). Yet, only a few studies on this topic have been reported, mostly based on a single species of pollen. Therefore, knowledge about factors affecting pollen rupture and PCGs release from allergenic pollen is needed for the management of allergic and asthmatic symptoms.

This study investigated the dynamics of pollen rupture and protein release upon contact with moisture using pollen from subtropical allergenic grass and weed species in Thailand (Bunnag et al. 2009), including *Sorghum halepense* (Johnson grass), *Urochloa mutica* (para grass), *Typha latifolia* (cattail), *Amaranthus hybridus* (green amaranth), and *Cyperus mitis* (nut grass). Three factors affecting pollen rupture and protein release were investigated: incubating solutions, incubation time, and time after shedding.

2 Materials and methods

2.1 Plant materials

Pollen from five species was used in this study: *Sorghum halepense* (Johnson grass), *Urochloa mutica* (para grass), *Typha latifolia* (cattail), *Amaranthus hybridus* (green amaranth), and *Cyperus mitis* (nut grass). These species were among the most prominent airborne pollen types based on a survey in Bangkok (Songnuan et al. 2015). These pollen species had also been shown to elicit allergic reactions among Thai atopic patients (*U. mutica*, *C. mitis*, and *A. hybridus*: Bunnag et al. 2009; *S. halepense* and *U. mutica*: Pacharn et al. 2019; *T. latifolia*: personal communications). Inflorescences of each species were collected from various sites around Bangkok metropolitan areas. Freshly cut inflorescences were arranged on soaked floral foam in a containing chamber. Shed pollen was gathered and separated from other plant parts by sieving. Only pollen shed within the first 24 h was used, partly as the 1-day-old sample, and the rest

was stored at room temperature and relative humidity ($\sim 70\%$) for 3- and 7-day-old samples. Due to the different capacity of pollen production of each species, the number of plants required for pollen collection varied from approximately 10 plants (for *T. latifolia*) to thousands (for the other species). Each pollen sample was inspected under a light microscope at $40\times$ magnification to ensure $> 95\%$ purity. Pollen size and wall thickness were measured and averaged from 100 grains. Pollen grain count was determined by suspending 1 mg of pollen in 1 ml water, and ten 1- μ l samples of the suspension were counted under the microscope.

2.2 Solutions for hydration

Solutions used in the experiments included artificial tears, distilled water, rainwater, normal saline (0.90% NaCl), and ethanol. Distilled water (pH 7) was obtained from a laboratory-grade water distillation system (Ultra Clear™ RO EDI, Siemens, Germany). Artificial tears contained 0.3% hydroxypropyl methylcellulose as an active ingredient (Tears Naturale II, ALCON, USA). Normal saline, pH 7 (General Hospital Products, Thailand), was purchased from a local pharmacy. Rainwater was harvested approximately 15 min after the rain started. To obtain representative rainwater, the water was pooled from five collecting times (pH 4–6) in June from the Faculty of Science, Mahidol University, in the heart of Bangkok. Rainwater was stored at 4 °C and filtered before use. Molecular-grade absolute ethanol was purchased from Merck, Germany. All solutions were kept at room temperature throughout experiments.

2.3 Pollen rupture analysis

Pollen suspensions were prepared by adding 1 ml of solution into 10 mg of a pollen sample in a microcentrifuge tube and inverting the tube gently about 2–3 times. At specified time points, 1 μ l of the suspension from each tube was mounted on a glass slide using 9 μ l of Calberla's solution as staining media and to preserve the status of pollen. Visibly ruptured and unruptured pollen grains were counted from all fields of a standard 22×22 mm cover glass under a light microscope at $400\times$ magnification. The counting time per slide was less than 1 min. Each condition was

tested in three tubes in tandem, and the experiment was repeated three times.

2.4 Quantification of proteins released from pollen

For each species, 10 mg of pollen was suspended in 1 ml of solution and mixed gently. Immediately, 10 μ l supernatant of the pollen suspension was collected into a separate tube as T_0 sample. For cumulative protein concentration analysis, the suspension was mixed gently before 10 μ l of the supernatant was sampled at each designated time point. For the analysis of protein accumulation during each time interval, most of the supernatant was removed at each time point and replaced with fresh solution and incubated until the next time point before the supernatant was replaced again in the same manner. Protein concentration in the supernatant collected from each time point was determined using Bradford's reagent (Bio-Rad, USA) using BSA as a standard.

2.5 SDS-PAGE analysis

Profiles of protein released from pollen were analyzed from collected supernatants kept at -20 °C. For each sample, 10 μ l was loaded into 14% SDS-PAGE gel. The gels were stained with Pierce® Silver stain kit (Thermo Scientific, USA) according to the manufacturer's instructions.

2.6 Statistical analysis

Statistical analysis was performed to determine the variation between the collected data using SPSS V.16 software. ANOVA analysis with Tukey post hoc test was used to estimate the variation in ruptured pollen percentage and released protein concentration. Pearson correlation coefficient analysis was used to determine the significance of the correlation between ruptured pollen percentage and released protein concentration of each species tested.

3 Results

3.1 Different dynamics of visible pollen rupture upon hydration

Five common subtropical allergenic grass and weed pollen species were selected for this study. Physical characteristics of these pollen species, including pollen size, weight, and pollen wall thickness, are shown in Table 1. After fresh (1-day-old) pollen grains were incubated in distilled water, all species showed rupture and released cellular contents, so-called pollen cytoplasmic granules (PCGs), that were readily observed as shown in Fig. 1.

As expected, the percentage of ruptured pollen grains increased as the incubation time in distilled water increased, for all species tested (Fig. 2). However, marked differences in the pollen rupture dynamics could be observed. At T_0 time point, approximately 70% of *C. mitis* pollen and 40–60% of the grass pollen (*S. halepense* and *U. mutica*) had ruptured. *C. mitis*, *S. halepense*, and *U. mutica* had a steady increase in ruptured pollen until almost all pollen grains were visibly ruptured in about 60 min. On the contrary, *A. hybridus* pollen grains were only about 20% ruptured at T_0 and up to 50% at T_{45-60} . The rupture of *A. hybridus* pollen plateaued at approximately 60%, even after an overnight incubation. *Typha latifolia* showed the lowest amount of visibly ruptured pollen at less than 20% at all time points.

3.2 Protein release from pollen upon hydration

To better understand the dynamics of protein release from each species, the concentrations of protein released into the supernatant were measured every 15 min with and without replacement of distilled water for periodic and cumulative released protein measurements, respectively. For all tested species, the highest concentration of released protein was found between 0 and 15 min after incubation and rapidly decreased after 15 min. At T_0 , *C. mitis* had the highest concentration of released protein from pollen at 119.47 µg/mg pollen, while *S. halepense* and *U. mutica* had similar concentrations of released protein at 104.96 and 104.51 µg/mg pollen, respectively (Fig. 3a). At T_{0-15} , the released protein concentrations of these three species increased to approximately 230 µg/mg pollen. On the other hand, *A. hybridus* pollen released only about 66 µg/mg protein at its peak time point (T_{0-15}). Compared to other species, *T. latifolia* pollen released the lowest, almost undetectable, amount of protein (< 30 µg/mg pollen). Even though the cumulative protein release increased with time in all species, the concentrations of released protein from these species were significantly different. The *C. mitis*, *S. halepense*, and *U. mutica* pollen released about three times more protein compared to the *A. hybridus* and *T. latifolia* pollen. Cumulatively, all species released 71–89% of total released protein within the first 30 min upon hydration (Fig. 3b).

Table 1 Physical characteristics of five pollen species in this study and amount of protein released upon hydration with distilled water (mean ± SEM)

	<i>T. latifolia</i>	<i>A. hybridus</i>	<i>S. halepense</i>	<i>U. mutica</i>	<i>C. mitis</i>
Pollen size (µm)	34.65 ± 3.66, 32.46 ± 1.76 (P/E ratio: 1.07)*	20.94 ± 1.78, 20.88 ± 1.64 (P/E ratio: 1.00)	32.18 ± 1.99, 31.92 ± 1.61 (P/E ratio: 1.00)	27.60 ± 1.13, 27.37 ± 1.08 (P/E ratio: 1.00)	27 ± 2.44, 20.27 ± 1.3 (P/E ratio: 1.33)
Pollen wall thickness (µm)	2.29 ± 0.49	1.20 ± 0.19	1.00 ± 0.15	1.11 ± 0.21	0.91 ± 0.14
Pollen grain count per milligram pollen (grains)	83.24 ± 3.35	148.14 ± 6.09	95.29 ± 2.27	110.76 ± 3.51	171.17 ± 5.85
Protein release per milligram pollen (µg/mg pollen)	150.9 ± 5.27	186.12 ± 2.01	503.42 ± 4.41	523.59 ± 5.92	584.56 ± 2.32
Protein release per grain (µg/grain)	1.81 ± 0.13	1.26 ± 0.11	5.28 ± 0.23	4.73 ± 0.22	3.42 ± 0.12

*P/E ratio, pollen polar diameter/pollen equatorial diameter

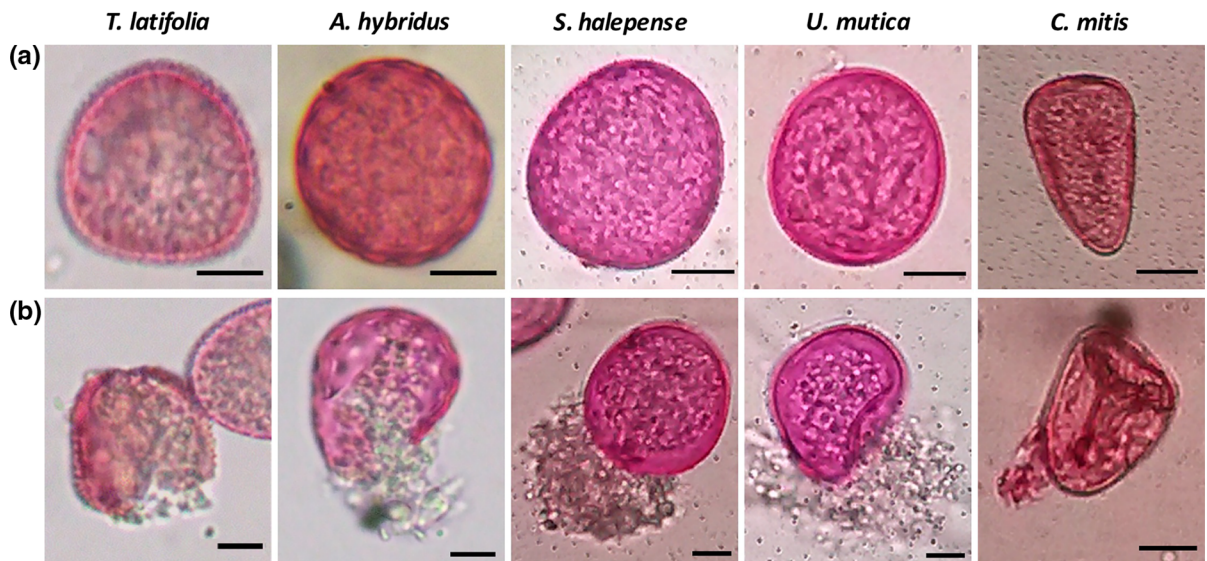


Fig. 1 Unruptured (a) and ruptured pollen (b) from five species: *Typha latifolia*, *Amaranthus hybridus*, *Sorghum halepense*, *Urochloa mutica*, and *Cyperus mitis* chosen for this study (scale bar = 10 μm)

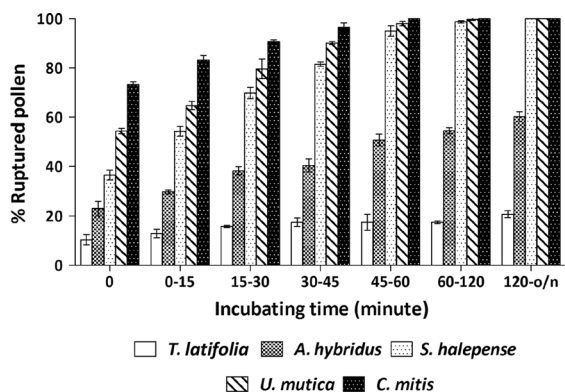


Fig. 2 Percentage of ruptured pollen from five species at different time points. Each value represents the mean of three independent experiments with the standard error of mean (SEM)

Because pollen size and weight vary from species to species, the amount of released protein per pollen grain was calculated. *T. latifolia* had the lowest number of pollen grains per milligram (~ 83 grains). Each grain of *T. latifolia* released about 1.81 μg of protein, which was also the lowest amount per grain (~ 15% of pollen grain weight). *A. hybridus* had 148 pollen grains per milligram; each grain released about 1.26 μg of protein. *S. halepense* and *U. mutica* had similar pollen grain weight and amount of released protein with about 95–110 grains per milligram, releasing about 4.7–5.2 μg of protein per grain. The

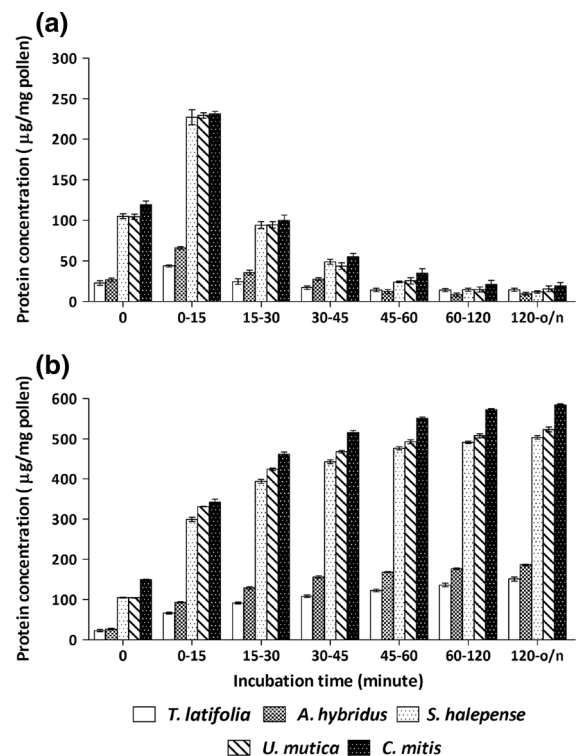
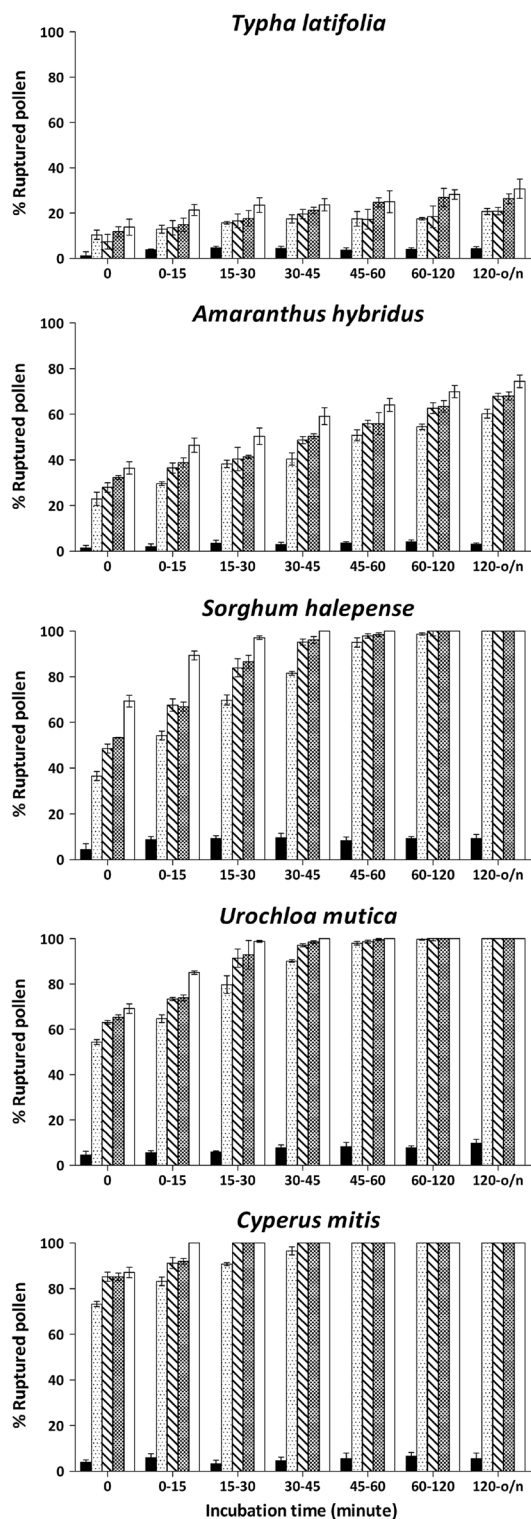
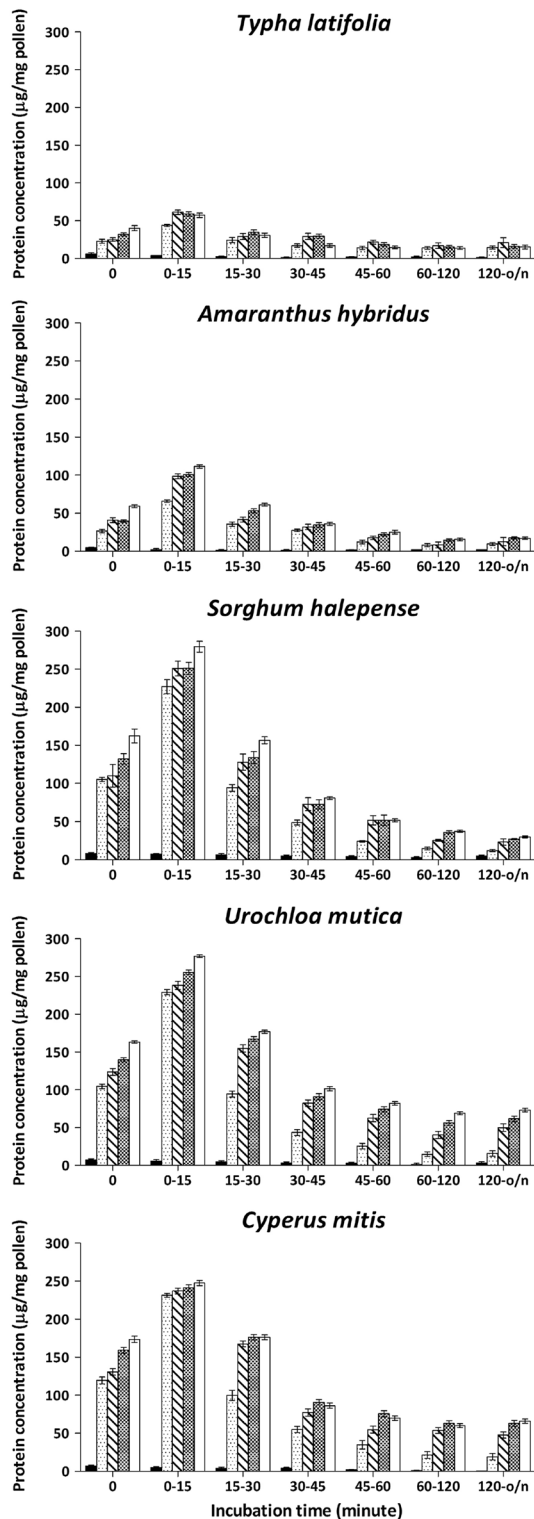


Fig. 3 Concentration of released protein at each 15-min interval (a) and cumulative released protein (b) from pollen of five species. Each value represents the mean of three independent experiments with the standard error of mean (SEM)

(a) Pollen rupture**(b) Released protein**

◀ **Fig. 4** Cumulative pollen rupture (a) and protein release (b) at each 15-min interval in various solutions (distilled water, normal saline, artificial tears, and rainwater). Ethanol was used as a negative control. Each value represents the mean of three independent experiments with the standard error of mean (SEM)

highest number of pollen grains per milligram was from *C. mitis* (171 grains), releasing 3.42 µg of protein per grain. Within the scope of this study, the amount of protein released seemed to correlate with the visible rupture, but not with pollen size. Correlation of ruptured pollen and protein release from different species of pollen was analyzed by Pearson's correlation analysis (mean 95% confidence interval $r = 0.887 - 0.996$). All species tested had high correlation of pollen rupture and released protein patterns.

3.3 Effects of hydrating solution on pollen rupture and protein release

For all species of pollen, incubation in artificial tears, rainwater, and normal saline triggered significantly more rapid and more extensive rupture than in distilled water. In most cases, rainwater caused the highest pollen rupture. Grass (*S. halepense* and *U. mutica*) and *C. mitis* pollen was about 50% ruptured at T_0 and increased to 100% within 2 h in all solutions. All incubating solutions could cause 20–40% rupture of *A. hybridus* pollen at T_0 and reached almost 75% rupture overnight. Nonetheless, no solution could rupture more than 30% of *T. latifolia* pollen, even after an overnight incubation (Fig. 4a).

The effects of incubating solutions on concentrations of released proteins were also investigated. Concentrations of protein released from pollen incubated in ethanol were not more than 60 µg/mg pollen. Overall, pollen incubated in artificial tears, rainwater, and normal saline released higher amounts of protein than in distilled water (Fig. 4b). The difference was most pronounced with grass (*S. halepense* and *U. mutica*) pollen in the first 30 min, with rainwater triggering the highest level of protein release.

3.4 Effects of pollen age on rupture and protein release

Because settled pollen could become airborne again by a gust of wind, we questioned whether the pollen

rupture and protein release would be affected by the age of pollen. To investigate the effect of pollen age, ruptured pollen and released protein were measured from fresh (no more than 1-day-old) and aged (3- and 7-day-old) pollen after exposure to distilled water. In all species tested and at all time points, fresh pollen had significantly higher rupture percentage than aged pollen. The pollen rupture was reduced about 50% after 7 days (Fig. 5a). The concentrations of protein released from pollen 3 days and 7 days after shedding were significantly lower than those from fresh pollen at all time points (Fig. 5b). The 3-day-old *C. mitis* pollen protein release was 60% less than that of fresh pollen. For the other tested species, 3- and 7-day-old pollen released approximately 30% and 60% less protein compared to fresh pollen.

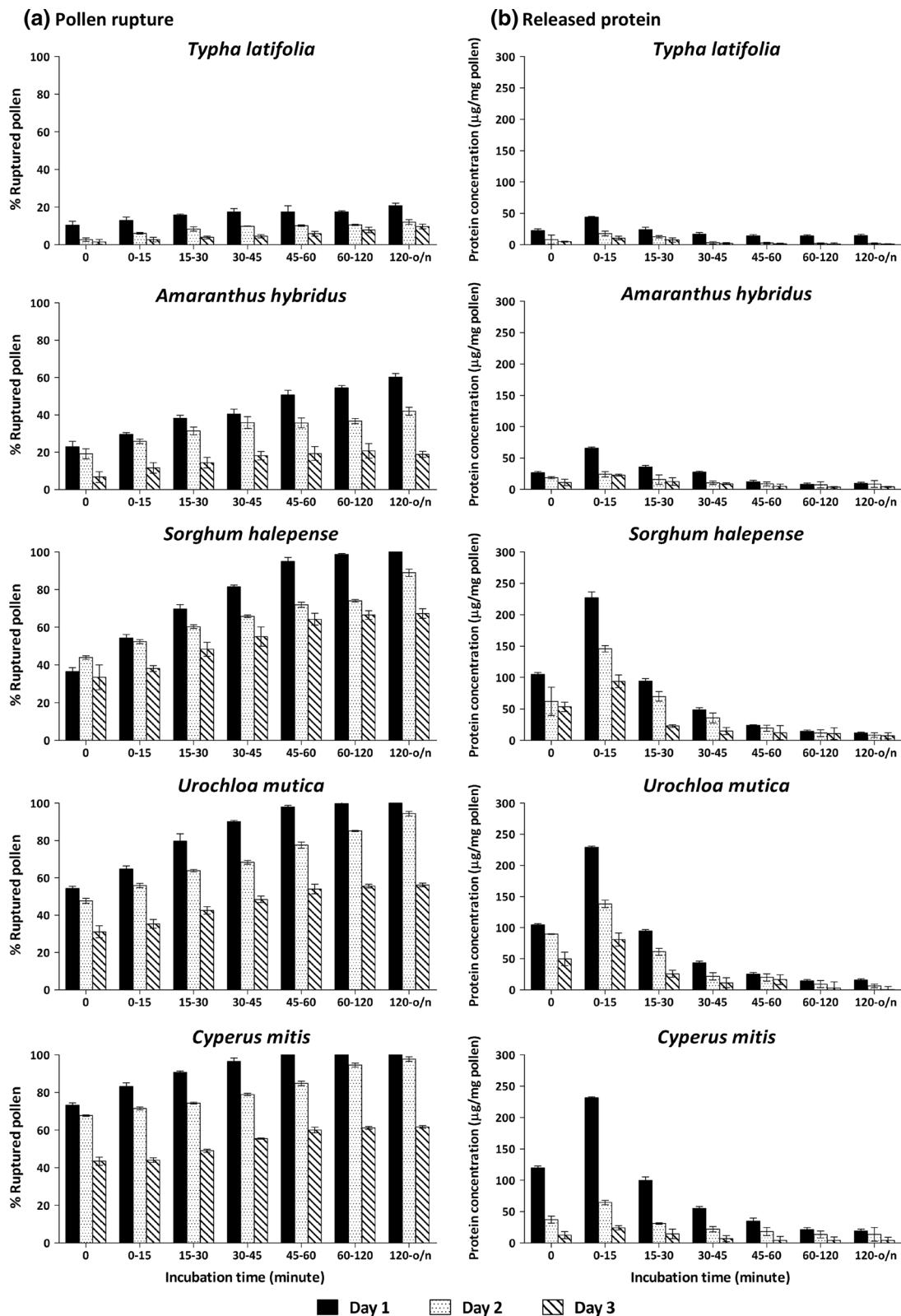
3.5 SDS-PAGE profiles of released proteins

SDS-PAGE analysis of fresh pollen showed that each species released proteins with a unique profile as shown in Fig. 6. *T. latifolia* and *A. hybridus* released only two major protein bands between 35 and 45 kDa. *S. halepense*, *U. mutica*, and *C. mitis* pollen released almost all protein components into water within the first 15 min. At T_{15-30} and after, very few faint bands could be detected. This indicated that the releasing time of each protein did not correlate with its size. When protein profiles of protein released into solution were compared to those of total pollen protein extract, there were several components present in the total protein extract that were absent in the released protein solution. Even though aged pollen released lower amounts of proteins compared to fresh pollen, the protein profiles were comparable (data not shown).

4 Discussion

This study demonstrated the different dynamics of pollen rupture and protein release upon hydration of pollen from five commonly found grass and weed species. It was found that the incubation time, time after shedding, and the incubating solution had significant impacts on these processes.

Visible rupture could be observed almost immediately after pollen came in contact with moisture. The majority of protein was released from pollen within 15 min after incubation. Similarly, birch pollen and



◀ **Fig. 5** Comparison of ruptured pollen (a) and released protein (b) at each 15-min interval from different ages after shedding (1 day, 3 days, and 7 days). Each value represents the mean of three independent experiments with the standard error of mean (SEM)

timothy grass pollen were previously shown to release detectable proteins into the supernatant within a few minutes (Grote et al. 1993; Vrtala et al. 1993). This time frame also correlates well with the results of pollen challenge studies that the symptoms start appearing within the first 30 min after pollen discharge (Jacobs et al. 2011, 2014). Interestingly, most protein was released within 30 min even though the percentage of ruptured pollen kept increasing from 0 to 60 min, suggesting that protein could be released even when pollen was not visibly ruptured.

When different species of pollen were compared, the grass and weed species had similar dynamics, with high percentages of ruptured pollen and large amounts of released protein at early time points. Little relationship between pollen size and visible protein release was found in this study. The *T. latifolia* pollen with thick pollen wall showed less rupture and less protein release. Thick walls could prevent moisture from entering pollen quickly and resist the explosive rupture, allowing only small amounts of protein to leak out through the aperture. However, this study was based on only a few species and many more species should be investigated before a general conclusion could be made.

We questioned whether pollen rupture and protein release would be affected by the incubation solutions, including distilled water, normal saline, artificial tears, and rainwater. Although all tested solutions caused considerable pollen rupture and protein release, rainwater was the most potent, especially in the first 30 min. The reason for the high efficiency of rainwater is unclear. It might be important to note that the rainwater used in this study was collected from an urban area and was found to have a low pH (pH 4.6) and may contain other toxic pollutants (Kulthanan et al. 2013). Normal saline and artificial tears could trigger pollen rupture as well as or better than distilled water, suggesting that this pollen could also rupture rapidly upon contact with ocular and nasal mucosa. However, real human tear and nasal secretion are complex mixtures of biologically active substances,

the role of which with respect to pollen rupture and allergenic protein release should be further studied. This is especially important since it has been known that patients with allergic rhinitis have altered nasal and conjunctival fluids and could be responsible for the priming phenomenon (Tomazic et al. 2014; Tomljenovic et al. 2016).

Interestingly, the two species of grass pollen remained quite potent after 7 days, compared to *C. mitis* pollen, which only released one-fourth of its protein 3 days after shedding. (Taylor et al. 2004) also observed that fresh pollen from birch was easily ruptured by moisture, but the commercial pollen was not ruptured. Another study demonstrated that commercial pollen of birch grass and ragweed were not efficiently ruptured after incubation in buffer (Gunawan et al. 2008). The ability of pollen to absorb moisture and germinate is likely to be optimized for each species over the course of evolution and could be related to other factors such as the pollen production capacity, pollen buoyancy, population density, and climate. After the time window is passed, the pollen aperture is less likely to absorb moisture. Therefore, the aged pollen of certain species is less likely to be ruptured and released proteins when incubated with water. For these reasons, patients who are exposed to fresh pollen are likely to have more severe symptoms. In addition, one should be cautious about using commercial pollen for clinical studies, especially when pollen rupture is involved such as in pollen challenge studies.

SDS-PAGE and silver staining analysis showed that most major protein components were released from pollen within 15 min upon contact with solutions. At later time points or when aged pollen was tested, released protein was different mostly in terms of quantity, not protein profiles. Other studies had previously shown that major allergenic proteins were released from pollen within minutes upon hydration (Grote et al. 2003; Taylor et al. 2002, 2004; Vega-Maray et al. 2006). Limited protein release from pollen had been shown to affect its ability to cause sensitization, such as bGST in birch pollen (Deiftl et al. 2014). In addition, ruptured pollen could generate oxidative stress via NAD(P)H oxidases that potentiate robust inflammation in the lower airways (Bacsi et al. 2006). The investigation of major allergen release was not possible with the current study because the major allergens had not been characterized for most of these

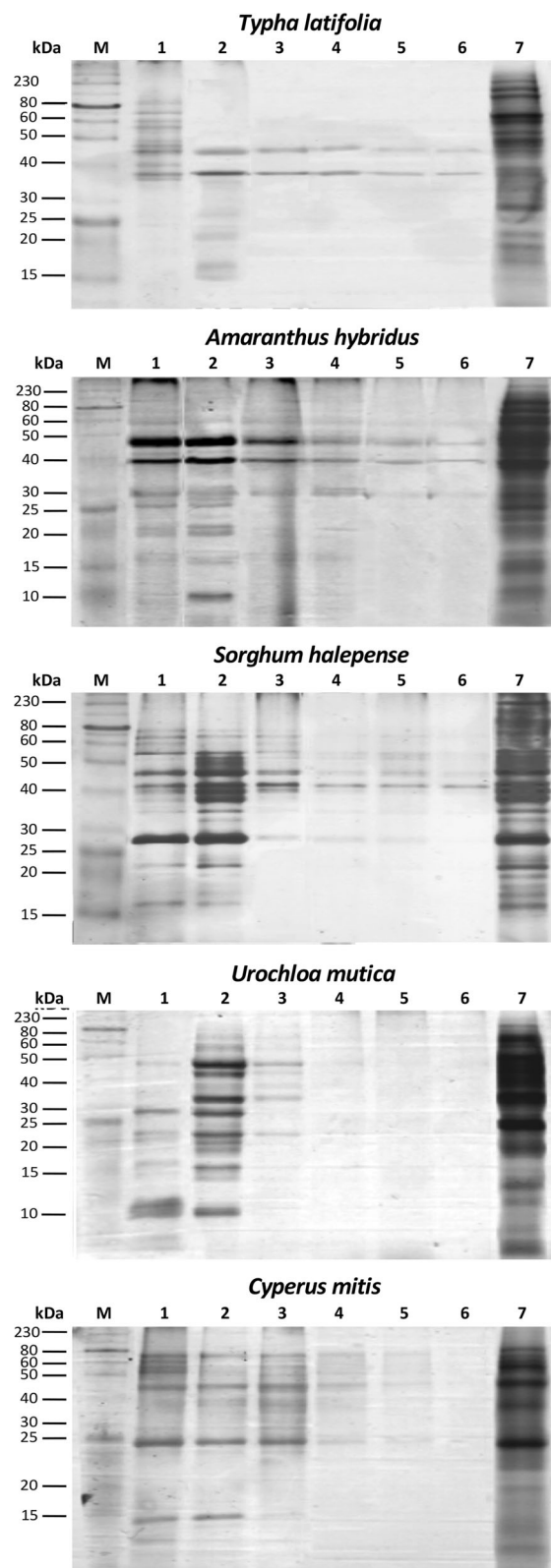


Fig. 6 Profiles of released proteins from pollen in distilled water. Protein was obtained from pollen 1 day after shedding, separated in SDS-PAGE gels and stained with silver staining; Lane M: protein marker; Lanes 1–6: released protein in distilled water at 0-, 0–15-, 15–30-, 30–45-, 45–60-, and 60–120-min intervals; Lane 7: total pollen extract with distilled water

species. In addition, several other factors had been shown to impact pollen rupture and protein release, including relative humidity, ozone, UV, temperature, and wind (Jacobs et al. 2014; Pasqualini et al. 2011; Schmidt 2016). The allergenic protein release in nature is undoubtedly much more complicated than could be replicated in laboratory.

This study partially explains why grass pollen is a potent allergen source. Not only does it rupture immediately upon contact with moisture, but it also releases high concentrations of protein. Moreover, aged pollen can still release a large amount of protein. On the contrary, *T. latifolia* pollen, which had been found abundantly in the airborne pollen survey, does not seem to be a significant cause of allergic rhinitis in the region (Songnuan et al. 2015). This is likely due to the small amount of protein released from the *T. latifolia* pollen. A previous study also showed that grass pollen simply ruptured after contract with rainwater, whereas tree pollen did not rupture although proteins could be released from the germination pore (Grote et al. 2003). The high potency of grass pollen is known as a significant cause of thunderstorm-related asthma (Newson et al. 1997).

Because of the variability in pollen rupture and protein release, combined with technical errors in pollen counting, this study supports that pollen monitoring could be less accurate at predicting allergic symptoms than direct measurement of airborne allergenic proteins (molecular aerobiology) as had been previously suggested in several other studies (Buters et al. 2015; Plaza et al. 2016). Furthermore, the amount of pollen protein released upon hydration could be a simple indicator, in addition to the number of airborne pollen count, to predict allergenicity of a given pollen species. This is particularly important for regions with year-round pollen season where the genuine sources of pollen allergy sensitization are still elusive and scores of pollen species must be tested. Further investigation in properties of airborne pollen, especially in relation to the increasing air pollution, could provide valuable

information with critical implication for pollen allergy management.

In conclusion, sedge and grass pollen was more than 50% ruptured instantaneously upon contact with moisture. Fresh pollen had more rupture and released more proteins than aged pollen. The highest amount of released proteins was found within the first 15 min after contact with moisture. *Cyperus mitis* had the highest percentage of pollen rupture and the highest concentration of released proteins, followed by *Sorghum halepense*, *Urochloa mutica*, *Amaranthus hybridus*, and *Typha latifolia*. Pollen incubated in rainfall water was damaged faster and released more proteins than in distilled water. Finally, incubation time, incubating solution, and age of pollen significantly affected the amount of pollen rupture and released proteins from allergenic pollen.

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