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Protocols

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Comparison of Pollen Grain Treatments Without Mechanical Fracturation Prior to Protein Quantification

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Abstract

Protein and amino acids in pollen are important nutritional components for larval development in several insect species, especially in Apoidea. The Bradford assay is a widely used method to measure relative protein content of pollen, which can shed light on pollen quality and consequences to fitness. Prior to using the Bradford assay, protein must be released from pollen grains, often using a mixture of chemical and mechanical fracturation methods. In this study, we tested the efficacy of protein extraction without using mechanical fracturation. We used pollen collected by the solitary bee *Osmia lignaria* Say to compare two known buffers associated with pollen protein analysis: phosphate-buffered saline and sodium hydroxide, and deionized water, and with different pollen weights from which we quantified protein using the Bradford assay. While all buffers and deionized water were useful in releasing protein from pollen grains collected by *O. lignaria*, the use of sodium hydroxide resulted in significantly higher protein quantification across all pollen weights. This methodological study can inform future studies of pollen nutrition in pollen-foraging species.

Key words: Bradford assay, sodium hydroxide, protein, pollen, spectroscopy

Protein found in pollen is a necessary nutritional requirement for bee larval development (Vaudo et al. 2015, Cane 2016). The nutritional value of pollen can differ among plants, and in heterogeneous landscapes, the effect of different plant pollen on bee development and fitness is an important avenue of research (Roulston et al. 2000, Vaudo et al. 2016, Nicholls and Hempel de Ibarra 2017). The Bradford assay is a useful method for assessing protein from biological sources, including plant pollen (Bradford 1976). This assay is based on the use of a dye that binds to free protein and fluorescing under light to produce a measurement of the amount of protein in a sample. The Bradford assay is relatively fast and capable of quantifying protein even in small samples (e.g., 1 mg; Vaudo et al. 2016). In contrast, other methods tend to be more labor- and time-intensive, and require a larger amount of sample (e.g., 1 g for use in micro-Kjeldahl; Ma and Zaga 1942, Buchmann 1986, Roulston et al. 2000). In studies involving pollen collected by solitary bees, especially when collected from heterogeneous landscapes, the amount of pollen that can be sampled can be a limiting constraint; thus, the Bradford assay is a useful approach in quantifying protein content of pollen in pollinator-based studies (Vaudo et al. 2016).

Pollen grains consist of an outer exine, which protects the innermost layer of cytoplasm containing protein and amino acids (Roulston and Cane 2000). Prior to the Bradford assay, the pollen grain must be treated to break down the exine and release protein into the treatment solution to bind to a fluorescent dye. Prior studies have reported on the use of chemical treatment and mechanical fracturation to expose the inner protein and established fracturation as a necessary method for obtaining protein content, such as using chloroform ($CHC1_3$) as a chemical treatment (Evans et al. 1991). Previous studies have used different chemical buffers alongside fracturation to prepare pollen for the Bradford assay, including phosphate-buffered saline (PBS) (Şahin et al. 2017) and sodium hydroxide (NaOH) (Vaudo et al. 2016), and deionized water (DW) (Avni et al. 2014), and different amounts of pollen, from 5 mg to 1 g. Each of these prior studies also varied in their laboratory techniques, such as using fracturation with a micropestle, temperature fluctuations, Mini Bead Beater, and different centrifugation or exposure times of pollen to the treatment. Centrifugation by itself should not alter the results of a Bradford assay unless samples are spun at extremely high speeds for long intervals. Contact with a strong or semi-strong acid treatment could influence the results of protein analyses with fracturation, whereas the length of contact with deionized water, which is considered stable, should not alter pollen exine chemistry unless combined with fracturation to promote fragility due to osmosis. Because different treatment substances vary in pH levels, there could be differences in the extent to which the exine is dissolved and the amount of inner protein that is released, regardless of fracturation.

Fracturation, the process by which pollen grains are broken or fractured to open the exine, can be a time-intensive process requiring

costly materials or expensive equipment. While the presence and absence of fracturation has been studied in plant pollen (Evans et al. 1991), the use of various chemical treatments alone as a means of protein release has not been previously reported. Moreover, studies of pollen protein are often based on pollen from a single plant species, rather than a mixed sample (Shahali et al. 2007, Russo et al. 2019). In generalist solitary bee species, protein content from mixed pollen samples must be considered to understand the consequences to bee health. A method of protein analysis for mixed pollen samples without fracturation could provide an inexpensive pathway for solitary bee researchers.

In this study, we used two buffers (PBS, NaOH) and DW and three pollen weights in a full factorial design when applied to mixed pollen samples collected by the blue orchard mason bee, *Osmia lignaria* Say (Hymenptera: Megachilidae). *Osmia lignaria* is native to western North America, and is a generalist pollinator visiting a range of taxa (Williams 2003). The primary goal of this study was to measure the effect of these different buffers and water, and without the use of fracturation methods, when applied to different amounts of *O. lignaria*-collected pollen on subsequent protein quantification levels as determined by the Bradford assay.

Experimental Design

We collected pollen from *O. lignaria* nests at three sites in late March 2017. The sites included a heavily urbanized residential location in Seattle, WA; the Washington Park Arboretum, which is located ~3 km northeast of downtown Seattle and covers 0.93 km^2 with >6,800 plant species and cultivated varieties from around the world; and the University of Washington Charles Lathrop Pack Experimental Forest, a 17.4 km^2 working forest with stands ranging in age from newly clear-cut to old-growth (~800 yr) located in rural Eatonville, WA. Each site contained >14 species of Angiosperms and a mix of native and non-native plants; the dominant Angiosperm species across all sites included the native species *Gaultheria shallon* and *Acer macrophyllum*, the non-native species *Crataegus monogyna*, and a mix of native and non-native species of *Salix*. All sites contained multiple species of *Rubus*, *Prunus*, *Pinus*, and *Stauntonia*. At each site, we placed a nesting box with 40 paper straws (diameter = 8mm) in which *O. lignaria* females oviposit and place a pollen provision. Following the completion of nesting activity in July 2017, straws were collected, and pollen samples were removed and stored at –80°C until processed (~8 wk).

Pollen samples at each site were homogenized using a mortar and pestle. We first conducted a pilot study to test the effect of drying pollen prior to protein quantification (Vaudo et al. 2016) and without fracturation. To do this, we randomly selected pollen samples from one randomly selected site, and weighed out 5, 10, or 20 mg samples (20 samples per weight for NaOH, and 10 samples per weight for PBS). In addition, we used replicates from the pilot study when using PBS and NaOH on dried and undried pollen. Half of the samples were then randomly chosen to be dried at 36°C for 24 hr, while the remaining samples were left undried. Because we did not detect a significant difference in protein quantification between dried and undried samples when using either the NaOH (t = –1.10; df = 11; P = 0.30) or PBS (t = –2.719; df = 5; P = 0.11) buffers, other than differences due to pollen weight, we used undried samples in subsequent analyses.

We then extracted 5, 10, and 20 mg samples from the homogenized pollen samples at each site. Samples were randomly assigned one of the two buffers or DW (0.01-M PBS, 0.1-M NaOH). In each experimental trial, we used 15 replicates from each site and for each buffer and water, and pollen weight combination. All experiments involved the homogenization of samples prior to analysis, including vortexing and mixture using a micropestle, for the purpose of resuspending settled pollen. Each experimental trial was repeated three times. Thus, across all sites, we used a total of 135 replicates for each buffer or water and weight.

Protocol

Extraction Methods for Comparison

DW

- 1.5 ml of DW was added to 5, 10, and 20 mg replicate pollen samples.
- Sample was vortexed for 20 s to precipitate solids and placed at ambient temperature (~ 20–22°C) for 24 h.
- Samples were centrifuged at 2,000 × *g* for 30 s. to precipitate solids, and 35 µl of substrate was transferred to a new tube.

PBS

- 1.5 ml of 1× 0.01-M PBS buffer (pH 8) was added to 5, 10, and 20 mg replicate pollen samples.
- Sample was vortexed for 20 s to precipitate solids and placed at ambient temperature for 24 h.
- Samples were centrifuged at 2,000 × *g* for 30 s to precipitate solids, and 35 µl of substrate was transferred to a new tube.

NaOH

- 1.5 ml 0.1-M NaOH was added to 5, 10, and 20 mg replicate pollen samples.
- Sample was vortexed for 20 s to precipitate solids and placed at ambient temperature for 24 h.
- Samples were centrifuged at 2,000 × *g* for 30 s to precipitate solids, and 35 µl of substrate was transferred to a new tube.

Bradford Protein Assay

We used the Bradford Assay with the Bio-Rad QuickStart Bradford Assay Kit (Catalog #5000201, Bio-Rad, Hercules, CA). Using the 1× dye reagent, we added 1. 5 ml Bradford dye to each 35 µl sample and vortexed. Samples were incubated in the dark for at least 25 min to ensure protein bound with the dye. We used a dilution series of QuickStart Bovine Serum Albumin (Bio-Rad, Hercules, CA) to initialize a standard curve. Absorbance readings at 595 nm were measured using a Genesys 30 visible spectrophotometer (ThermoFisher, Waltham, MA). We tested the effect of the treatment buffers (NaOH, PBS) on the BSA standard quantification, and observed a common slope (t = 0.07; P = 0.95) and intercept (t=-0.56; P = 0.59), indicating no differences on protein quantification between the buffers. We ran a blank sample prior to each spectrophotometer analysis.

For statistical analyses, protein concentrations were transformed using $\log_{10}$ to normalize the distribution. Pollen collection sites were considered a random effect to account for site variation. We tested the main effects of treatment (PBS, NaOH, DW), pollen weight (5, 10, 20 mg), and their interaction in a linear mixed-effects model using lme4 (Bates et al. 2015) in R (R Core Team 2018). P-values for main and interaction effects were estimated using lmerTest based

on a Type III analysis of variance and the Satterthwaite method for estimating degrees of freedom. Post-hoc tests were also conducted in lmerTest based on least squares means (Kuznetsova et al. 2017).

Results

We incorporated some common steps for use with each buffer and water to minimize differences among them. A prior application of the PBS buffer included centrifugation at 14,000 × *g* for 3 min at 4°C (Şahin et al. 2017). However, in initial testing, we observed no differences in final protein quantification when using 2,000 × *g* for 30 s without refrigeration. A prior application of the NaOH buffer (Vaudo et al. 2016) included grinding in a few drops of NaOH buffer, pre-treatment, and centrifugation to remove debris; we excluded this step to avoid fracturation as this study included grounding with a micropestle along with NaOH drops. We also used a standardized 24 hr treatment period in all experiments.

We observed a significant effect of buffers and water ($F = 486.7$; df = 2, 532; $P < 0.001$) and pollen weight ($F = 1128.4$, df = 2, 532; $P < 0.001$), but no significant interaction effect ($F = 3.1$; df = 2, 532; $P = 0.043$). Across buffers and water, protein concentration significantly increased with increasing pollen weight (Table 1, Fig. 1). Across all pollen weights, NaOH resulted in significantly higher protein quantification in Bradford assay than DW and PBS, which did were not significantly different from each other (Table 1, Fig. 1).

Table 1. Mean (±SE) concentration of protein (μg/ml) by pollen weight, and for the two buffers (NaOH and PBS) and deionized water (DW)

	Pollen weight		
	5 mg	10 mg	20 mg
NaOH	561.99 (12.62)a	919.24 (19.89)a	1470.92 (25.97)a
PBS	122.37 (4.24)b	214.53 (9.91)b	383.00 (14.04)b
DW	97.54 (5.54)b	158.46 (9.35)b	270.08 (15.53)b

Within each pollen weight, different letters represent significant differences ($\alpha < 0.05$).

Discussion

The pollen exine serves to protect the innermost contents of the pollen grain, including the genetic material necessary for reproduction. The outermost level of the exine is covered with a pollen coat commonly containing a variety of proteins, lipids, and pigments. The use of a strong base such as NaOH, which can chemically break the exine without fracturation, resulted in the highest measurement of total protein from pollen samples (Fig. 1). Coupled with its relatively low cost, the use of NaOH would be recommended as a buffer treatment without fracturation prior to the Bradford assay in this study system. It is also intuitive that larger pollen samples will produce higher protein quantification relative to smaller pollen samples with successful exposure of protein content. In small pollen samples (e.g., 5 mg), the use of NaOH is particularly important given the fairly low protein quantification when using DW or PBS (Table 1, Fig. 1). This study adds to our knowledge by comparing three treatments under similar study conditions and when using different-sized mixed-pollen samples, without the use of mechanical fracturation.

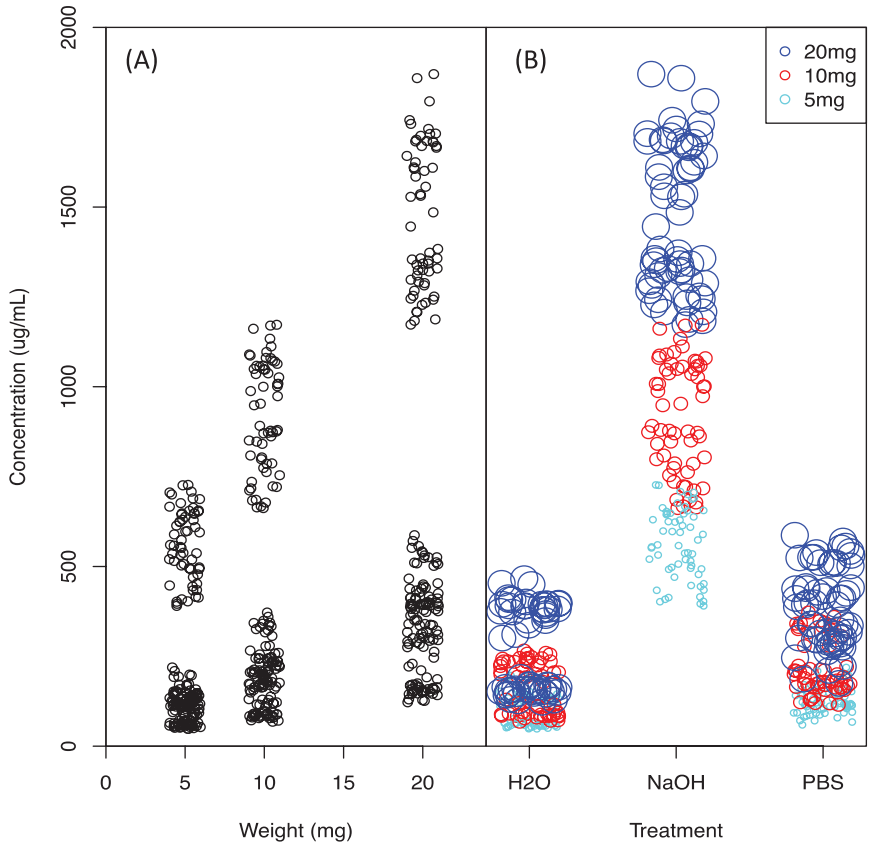

Fig. 1. Concentration of protein by pollen sample weight (A) and treatment (B).

A potential limitation is that the dye used in the Bradford assay is blue in its anionic form, and thus any use of an anionic base, such as NaOH, could result in false positive fluorescence at absorbance measured at 595 nm. Also, the standard curve used for the Bradford assay with a spectrophotometer is based upon NaOH; however, a molar concentration of NaOH ≤0.1-M would minimize false positive fluorescence and reduce the probability of inaccurate protein quantification. Another limitation is that because pollen was field-collected, which is a common approach in field-based pollinator ecology studies, it is not possible to determine the proportion of total protein extracted by any of these techniques. However, we note that all techniques resulted in protein identification, suggesting that protein was present at varying levels within pollen samples.

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Author Contributions

LRW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing; PCT: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

References Cited

Avni, D., H. P. Hendriksma, A. Dag, Z. Uni, and S. Shafir. 2014. Nutritional aspects of honey bee-collected pollen and constraints on colony development in the eastern Mediterranean. J. Insect Physiol. 69: 65–73.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Soft. 67: 1–48.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Buchmann, S. L. 1986. Vibratile pollination in Solanum and Lycopersicon: A look at pollen chemistry. W.G. D'Arc. Columbia University Press, New York, NY.

Cane, J. H. 2016. Adult pollen diet essential for egg maturation by a solitary Osmia bee. J. Insect Physiol. 95: 105–109.

Evans, D. E., P. E. Taylor, M. B. Singh, and R. B. Knox. 1991. Quantitative analysis of lipids and protein from the pollen of *Brassica napus* L. Plant Sci. 73: 117–126.

Kuznetsova, A., P. B. Brockhoff, R. H. B. Christensen, and S. P. Jensen. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. J. Stat. Soft. 82: 1–26.

Ma, T. S., and G. Z. Zaga. 1942. Micro-Kjeldahl Determination of Nitrogen. A New Indicator and An Improved Rapid Method. Industrial and Engineering Chemistry - Analytical Edition 14: 280–282.

Nicholls, E., and N. Hempel de Ibarra. 2017. Assessment of pollen rewards by foraging bees. Funct. Ecol. 31: 76–87.

Roulston, T. H., and J. H. Cane. 2000. Pollen nutritional content and digestibility for animals. Plant Systems and Evolution 222: 187–209.

Roulston, T. H., J. H. Cane, and S. L. Buchmann. 2000. What governs protein content of pollen: pollinator preferences, pollen–pistil interactions, or phylogeny? Ecol. Monogr. 70: 617–643.

R Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Russo, L., A. D. Vaudo, C. J. Fisher, C. M. Grozinger, and K. Shea. 2019. Bee community preference for an invasive thistle associated with higher pollen protein content. Oecologia. 190: 901–912.

Şahin, A. A., İ. Erdoğan, Ş. Alan, and N. M. Pınar. 2017. Comparison of pollen protein patterns and pollen morphology of *Taxus baccata* L. and *Cupressus arizonica* Greene. Int. J. Nat. Life Sci. 1:83–88.

Shahali, Y., A. Majd, G. Tajaddod, Z. Pourpak, M. Haftlang, and M. Moein. 2007. Comparative studies of the pollen protein contents in two major varieties of Cupressus arizonica planted in Tehran. Iranian Journal of Allergy, Asthma and Immunology (IJAAI). 6: 123–127. https://www.sid.ir/en/journal/ViewPaper.aspx?id=95178

Vaudo, A. D., J. F. Tooker, C. M. Grozinger, and H. M. Patch. 2015. Bee nutrition and floral resource restoration. Curr. Opin. Insect Sci. 10: 133–141.

Vaudo, A. D., H. M. Patch, D. A. Mortensen, J. F. Tooker, and C. M. Grozinger. 2016. Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. Proc. Natl. Acad. Sci. U.S.A. 113: E4035–E4042.

Williams, N. M. 2003. Use of novel pollen species by specialist and generalist solitary bees (Hymenoptera: Megachilidae). Oecologia 134: 228–237.