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Extraction of sporopollenin exine capsules from sunflower pollen grains†

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Sporopollenin exine capsules (SECs) are highly robust natural microscale capsules that can be extracted from plant spores and pollen grains, albeit through complex processing schemes. Herein, we report new insights into pollen processing by alkaline lysis and acidolysis with various process conditions. Alkaline lysis of sunflower pollen grains damages the unique pollen microstructure and acidolysis enables us to devise a simple process to extract SECs from sunflower pollen grains with a uniform particle size distribution. The SECs retain the natural morphology, offering an improved general scheme to streamline pollen processing for biomaterial applications.

1. Introduction

Natural microcapsules produced from plant spores and pollen grains have received increasing attention in recent years due to their broad utility as drug delivery vehicles, cell encapsulating scaffolds and biotemplates for advanced materials synthesis.[1–7] Conventional manufacture of polymeric microcapsules is costly and difficult, especially to obtain microcapsules with a uniform size distribution and large inner cavity.[8–10] There is wide interest in producing microcapsules through alternative strategies, and pollen-based microcapsules offer a natural solution that is readily available in abundant quantities.[1,11–13] Pollen constituents are mainly protected by a cellulose-rich cell wall called the intine, and a resistant outer wall composed largely of sporopollenin called the exine. Exine morphology varies significantly between pollen species, with highly unique and reproducible 3D architectures and morphologies. Importantly, the exine also provides environmental protection for successful pollination, motivating its extraction for use as natural microcapsules which are generally recognized as safe by the US Food and Drug Administration (FDA).[14,15] Sporopollenin exine capsules (SECs) produced from spores and pollen grains are resistant to extreme temperatures, pressures, and harsh chemical treatments as well as are devoid of allergenic materials.[1,14] In order to extract SECs, a simple chemical process is highly desirable to remove all interior and surface pollen constituents without altering the native microstructure of the exine capsule.[12] Several attempts have reported the extraction of SECs from various plant spores and pollen grains by complex multiple steps including alkaline lysis, acidolysis, and enzymatic processes.[1,2,13,16] While various degrees of success have been achieved in obtaining intact SECs without pollen inner constituents, in all cases, the process is time consuming and requires harsh reflux conditions which are particularly challenging when working with highly ornamented 3D microstructures. The primary requirement of the extraction process is to achieve empty SECs without appreciable damage to the intrinsic exine surface morphology. In this regard, sunflower pollen grains stand out due to their highly ordered surface spike ornamentation and monodisperse size distribution, which provide motivation for the development of novel 3D materials and drug delivery platforms.[1,4,17]

To address this issue, the objective of the current study is to develop a simple chemical scheme to extract SECs from sunflower pollen grains. In particular, we report an extraction scheme to isolate sunflower SECs by a simple chemical process that involves acidolysis using phosphoric acid, resulting in the successful extraction of sunflower SECs which retain highly ornamented microstructures. The processed SECs were rigorously characterized by CHN elemental analysis, scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and dynamic imaging particle analysis (DIPA). The reported method represents a process improvement over existing strategies and builds on chemical knowledge to extract SECs in order to establish a general scheme that can be broadly applied.

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2. Experimental

2.1. Materials

Defatted sunflower pollen grains (*Helianthus annuus* L.) were procured from Greer labs (NC, USA). Potassium hydroxide, hydrochloric acid, Tween-20, phosphoric acid (85% w/v), reagent grade salts and solvents were procured from Sigma-Aldrich (Singapore). Polystyrene microspheres (50 ± 1 μm) were purchased from Thermoscientific (CA, USA). Vectashield (H-1000) medium was procured from Vector labs (CA, USA) and sticky-slides, D 263 M Schott glass, No. 1.5H (170 μm, 25 mm × 75 mm) unsterile were procured from Ibidi GmbH (Munich, Germany). Milli-Q water (Millipore Corp., MA, USA) with a resistivity of 18 MΩ cm was used in all the experiments.

2.2. Extraction of sunflower sporopollenin exine capsules (SECs)

The natural sunflower pollen grains were collected from the Czech Republic by Greer labs (NC, USA) and were harvested by vacuum and then allowed to water set. The cleaning of pollen grains to remove plant debris was performed by mechanical vibration under vacuum. The cleaned pollen grains were chemically washed using acetone at room temperature and defatted by washing with ACS grade ethyl ether and supplied as defatted sunflower pollen grains. In our current extraction process, we have used defatted pollen grains as the starting material to produce sunflower SECs. The sunflower SECs were extracted by three different approaches. (1) Alkaline lysis: in this process, defatted sunflower pollen grains (20 g) were suspended in a round bottom perfluoroalkoxy (PFA) flask fitted with a glass condenser. Alkali (6% potassium hydroxide, 100 ml) was added to the flask and gently mixed to form a homogeneous suspension and heated at 80 °C for 12 hours under gentle stirring (replenished with fresh alkali after 6 hours). The sporopollenin after alkaline lysis was collected by centrifugation at 4500 rpm for 5 min and washed twice using hot water (50 ml), hot ethanol (50 ml) and dried at 60 °C for 8 hours. (2) Acidolysis using 6 M hydrochloric acid (HCl): defatted sunflower pollen grains (2 g) were suspended in 15 ml 6 M HCl and heated at 70 °C under gentle stirring for 48 hours, HCl was removed after 24 hours by filtration and replenished with fresh 6 M HCl. Collected SECs were washed using Milli-Q water and ethanol by gentle stirring and dried in an oven at 60 °C for 8 hours. In order to reduce HCl acidolysis processing time, the heating time was reduced to 10 hours, 20 hours and 30 hours and extensive washing was implemented using hot water (5 × 50 ml), hot acetone (2 × 50 ml), hot 2 M hydrochloric acid (50 ml), hot water (5 × 50 ml), hot acetone (50 ml) and hot ethanol (2 × 50 ml). SECs were dried at 60 °C for 8 h and stored in a dry cabinet until further characterization. To remove surface-adhered proteinaceous materials, sunflower SECs after HCl-acidolysis (10 h) were washed with trypsin (0.25%) or Tween-20 (2%) separately for 24 hours and further washed using sodium chloride (1%) and water. Collected SECs were dried in an oven at 60 °C for 8 hours. (3) Acidolysis using phosphoric acid (85% v/v) : defatted sunflower pollen grains (2 g) were suspended in phosphoric acid (H_3PO_4 15 ml) and mixed gently to form a homogeneous suspension. The pollen grains were heated at 70 °C under gentle stirring for 10 hours. The SECs were collected by filtration and washed extensively by using hot water (5 × 50 ml), hot acetone (2 × 50 ml), hot 2 M hydrochloric acid (50 ml), hot water (5 × 50 ml), hot acetone (50 ml) and hot ethanol (2 × 50 ml). SECs were dried at 60 °C for 8 hours and stored in a dry cabinet at 25 °C until further characterization.

2.3. Elemental analysis

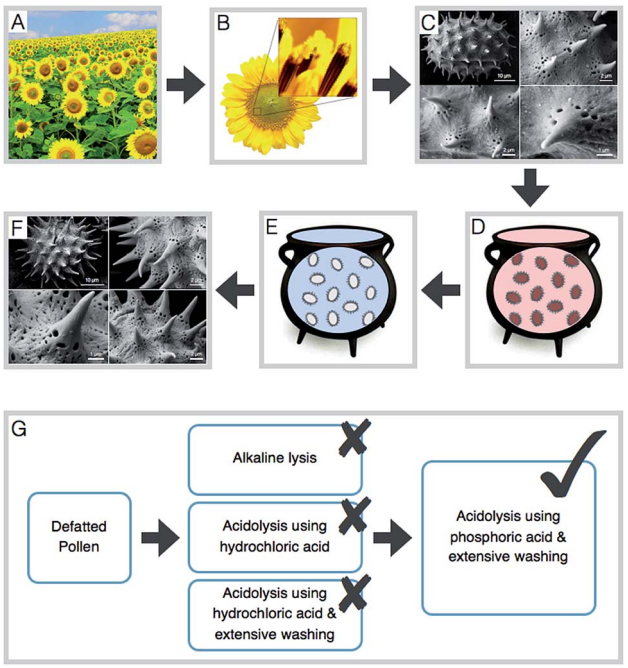
Defatted sunflower pollen grains and SECs produced using different chemical process were analyzed by using a calibrated Vario EL III CHN elemental analyzer (Elementar, Hanau, Germany). Unprocessed and processed samples were dried at 60 °C for at least 1 hour before CHN analysis, and all measurements were done in triplicate. Final protein concentration in unprocessed and processed samples was measured using percent nitrogen with an assumed multiplication factor of 6.25 to convert weight percent of nitrogen to weight percent of protein.[1]

2.4. Dynamic image particle analysis (DIPA)

Dynamic image particle analysis by FlowCam®: a bench top system (FlowCamVS®, Fluid Imaging Technologies, Maine, USA) was equipped with a 200 μm flow cell (FC-200), a 20× magnification lens (Olympus®, Japan) and controlled by the visual spreadsheet software version 3.4.11. The system was flushed with 1 ml deionized water (Millipore, Singapore) at a flow rate of 0.5 ml min^{-1} and flow cell cleanliness was monitored visually before each sample run. Defatted sunflower pollen grains, SECs (2 mg ml^{-1}) with a pre-run volume of 0.5 ml were primed manually into the flow cell and were analyzed with a flow rate of 0.1 ml min^{-1} and a camera rate of 10 frames per s. A minimum of 10 000 particles were fixed as the count for each measurement and three separate measurements were performed. The DIPA was carried out using 1000 highly focused particles segregated by edge gradient. The instrument was calibrated using polystyrene microspheres (50 ± 1 μm).[11] Representative data was plotted as a spline curve fitted to histogram data and values were reported with standard deviations.

2.5. Confocal laser scanning microscopy analysis

Fluorescence from defatted sunflower pollen grains and SECs processed using different methods were analyzed by confocal laser scanning microscopy (Carl Zeiss LSM700, Jena, Germany) equipped with three spectral reflected/fluorescence detection channels, six laser lines (405/458/488/514/561/633 nm), and a Z1 inverted microscope (Carl Zeiss, Germany).[11] Samples were mounted on sticky slides (Ibidi, Germany) then a drop of mounting medium (Vectashield®) was added before covering with another sticky slide. Images were collected immediately under the following conditions: laser excitation lines 405 nm (6.5%), 488 nm (6%) and 561 nm (6%) with DIC in an EC Plan-Neofluar 100× 1.3 oil objective M27 lens. Fluorescence was collected in photomultiplier tubes equipped with the following emission filters: 416–477, 498–550, 572–620. The laser scan



Scheme 1 Extraction process to isolate intact and clean sunflower sporopollenin exine capsules (SECs) while conserving distinctive sunflower pollen morphology: schematic shows (A) and (B) large scale cultivation of sunflowers and the location of sunflower pollen. (C) Scanning electron microscopic images of defatted pollen grains with different magnifications. (D) SEC extraction processes involving alkaline lysis with KOH, acidolysis with HCl or H_3PO_4 to remove all pollen constituents. (E) Washing steps using organic solvents and dilute acids to remove residual pollen materials to yield clean, intact SECs. (F) Scanning electron microscopic images of SECs after complete processing at various magnifications. (G) Different extraction approaches used to isolate intact sunflower SECs.

speed was set at 67 seconds per each phase (1024 × 1024: 84.94 μm^2 sizes) and plane mode scanning with a pixel dwell 12.6 μseconds. The iris was set as optimal for the sample conditions and all images were captured at the mid-region of the particle. All other settings were fixed for all samples, and per sample, at least three images were captured and processed using ZESS 2008 software (ZEISS, Germany).

2.6. Surface morphology evaluation by scanning electron microscopy (SEM)

SEM imaging was performed using a FESEM 7600F (JEOL, Japan). Cross section samples were prepared by mounting intact sunflower pollen grains or SECs on SEM sample mounting tape and then immersing in liquid nitrogen for about 20 to 30 seconds. A steel scalpel blade (No. 10 RS Components, Singapore) was used to slice across the SECs several times. The cut lines were followed during SEM until a suitable cracked SEC was found. Samples were coated with platinum at a thickness of 10 nm by using a JFC-1600 (JEOL, Japan) (20 mA, 60 s). Images were recorded by using FESEM with an acceleration voltage of 5.00 kV at different magnifications to observe morphological changes of defatted sunflower pollen grains and processed SECs.

3. Results and discussion

Scheme 1 depicts the extraction process for sunflower SECs. The sunflower pollen grains are readily obtained from field harvests and are separated from miscellaneous plant debris (Scheme 1A and B). Sunflower pollen possesses a unique microstructure with a uniform size distribution (37 ± 0.21 μm) and surface decorated with spikes surrounded by pores as imaged by SEM

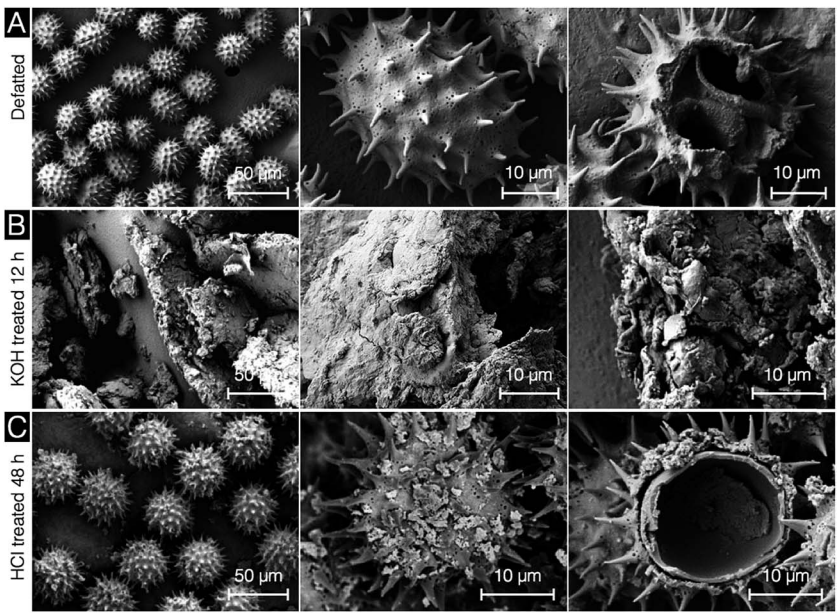


Fig. 1 Scanning electron microscopic images of sunflower pollen grains and sporopollenin exine capsules (SECs) obtained from different extraction processes: (A) defatted sunflower pollen grains. (B) SECs after alkali (6% potassium hydroxide) treatment (12 hours). (C) SECs after acidolysis using hydrochloric acid (6 M) process for 48 hours.

(Scheme 1C). The pollen grains are processed under gentle heating conditions to remove all pollen constituents and washed extensively with a series of aqueous and organic solvents to remove residual pollen constituents (Scheme 1D and E). The SEM images indicate that sunflower SECs retain the pollen intrinsic microstructure and have a uniform size distribution (30 ± 0.4 μm) (Scheme 1F). We first strategized our sunflower SECs extraction based on alkaline lysis with 6% potassium hydroxide (KOH) or acidolysis using 6 M hydrochloric acid (HCl) at 70 °C (ref. 18 and 19) (Scheme 1G). The SECs isolated from alkaline lysis are completely damaged and lose their unique spiky microstructure, and the SECs isolated by acidolysis (HCl) processing contain significant amounts of residual proteinaceous material after HCl processing only or after additional washing using water, solvents, trypsin (0.25%) or Tween-20. Hence, we finally extracted sunflower SECs by acidolysis using phosphoric acid (85% v/v) with extensive water and solvent washing, and the resulting isolated SECs are clean with an intact microstructure.

The representative SEM images of sunflower pollen grains before and after alkaline lysis and HCl acidolysis are presented in Fig. 1. Defatted sunflower pollen grains possess uniform spikes on the pollen surface and cross-section analysis reveals the presence of pollen constituents inside the large inner cavity (Fig. 1A). Interestingly, the pollen microstructure is damaged by alkaline lysis resulting in the loss of the unique microstructure (Fig. 1B). On the other hand, acidolysis using HCl was able to retain the microstructure, yet extracted debris still remained on the SEC surface (Fig. 1C).

In order to achieve empty, clean SECs devoid of all pollen constituents, we examined the effect of HCl acidolysis duration with a cleaning process using a series of organic solvents and dilute acids. Fig. 2A–C show the SEM images of sunflower SECs after 10 hours, 20 hours and 30 hours HCl acidolysis. The washing steps improved the cleanliness of capsules with less pollen debris and the SECs are morphologically intact. However, cross-section SEM images reveal residual pollen constituents inside the SEC cavity. It is crucial to remove all pollen constituents from sunflower SECs to create a large inner cavity devoid of allergenic proteins. Therefore, we incorporated additional washing steps using trypsin and Tween-20 (ref. 20) Fig. 2D demonstrates that trypsin incubation induced breakage

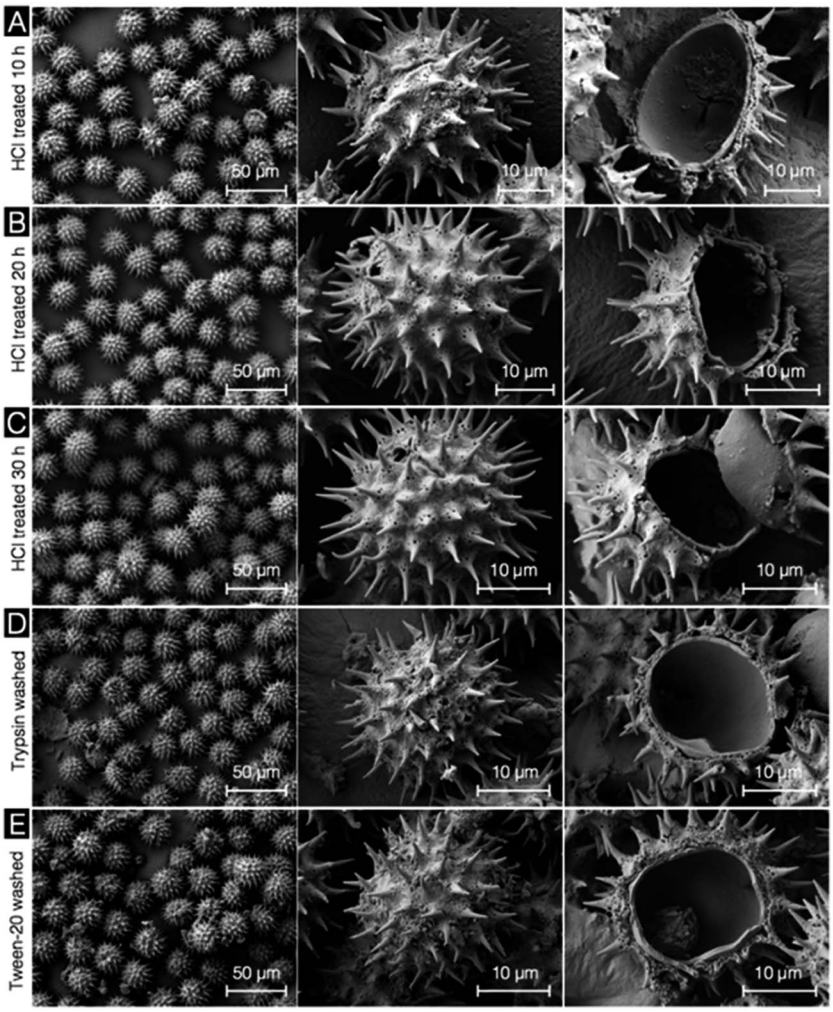


Fig. 2 Scanning electron microscopic images of sunflower sporopollenin exine capsules (SECs) after acidolysis using hydrochloric acid and extensive washing for different periods of time: (A) 10 hours. (B) 20 hours. (C) 30 hours. (D) Trypsin washed SECs. (E) Tween-20 washed SECs.

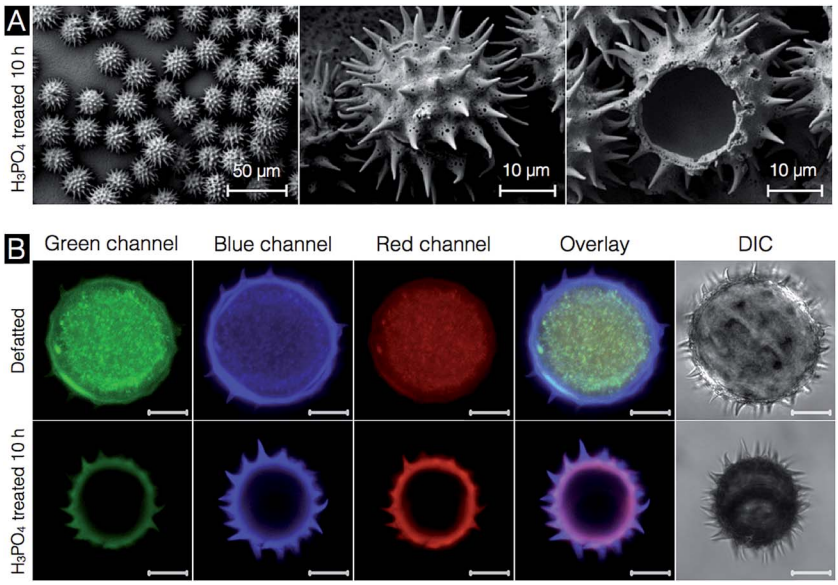


Fig. 3 Sunflower sporopollenin exine capsules (SECs) produced after the extraction process using phosphoric acid: (A) scanning electron microscopic images of SECs after acidolysis at different magnifications and a cross-section image. (B) Confocal microscopy analysis of defatted sunflower pollen grains before and after the extraction process: CLSM images in the first row are defatted pollen grains before processing and indicate autofluorescence due to the presence of terpenoid, phenolic, and carotenoid molecules. Second row images indicate clean SECs with complete removal of pollen constituents (scale bars are 10 μm).

of SECs spikes although there was a relatively clean inner cavity. With Tween-20 washing, the sunflower SECs still retained pollen debris on the surface, indicating that residual pollen debris may include a cellulosic material along with pollen proteins (Fig. 2E).[14,16]

As phosphoric acid is more efficient in the removal of cellulose materials from spores and pollen,[1,13,16] we next investigated acidolysis treatment of sunflower pollen grains by using phosphoric acid (85% v/v) as an extraction process. The SEM images of sunflower SECs after acidolysis using phosphoric acid showed intact clean SECs (Fig. 3A). It is noteworthy that the surface ornamentation of the produced SECs resembles that of native sunflower pollen grains. In order to confirm the removal of pollen constituents from SECs, we analyzed the sunflower pollen grains before and after the extraction process by CLSM. It is well known that pollen grains exhibit autofluorescence due to their inner constituents.[21,22] The sunflower pollen exhibits strong autofluorescence before the extraction process (Fig. 3B) while, in marked contrast, no autofluorescence was observed inside the SECs after acidolysis with phosphoric acid, suggesting successful removal of inner constituents. Our CLSM data also supports that the acidolysis using phosphoric acid (85% v/v) produces intact capsules, albeit somewhat smaller than the unprocessed samples due to removal of pollen cellular constituents.[14] The reduction in SEC size is mainly due to a loss of hydration resulting from complete removal of cytoplasmic materials.[23] Incidentally, it is interesting to note that in nature, pollen grains change shape, volume and structure due to pollen hydration, dehydration and rehydration as part of the biological processes involved in plant reproduction.[23,24]

In order to verify the reduction in particle size due to phosphoric acid acidolysis, we also performed high-throughput particle characterization of defatted pollen grains and SECs by using dynamic imaging particle analysis (DIPA). The results indicate that the average diameter of defatted sunflower pollen grains is 37 ± 0.2 μm with a narrow size distribution (Fig. 4A). Upon phosphoric acid acidolysis, the SEC average size is reduced to 30 ± 0.4 μm, supporting our CLSM data that shows a reduction in SEC diameter after removal of pollen constituents (Table S1†).[23–25] DIPA images of untreated sunflower pollen reveal a unique microstructure with surface ornamentation consisting of spikes (Fig. 4B). After the extraction process with phosphoric acid acidolysis, the sunflower SECs retained the native microstructure without any damage to the SEC structure (Fig. 4C). Taken together, the data supports that the phosphoric acid extraction process yields SECs with uniform size and

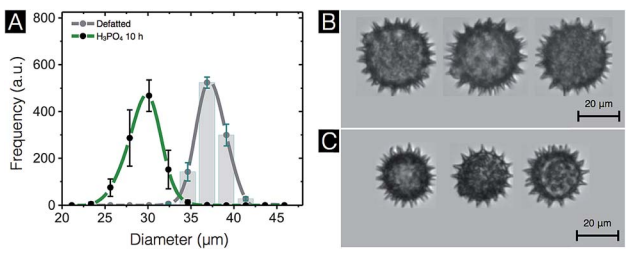


Fig. 4 Characterization of sunflower sporopollenin exine capsules (SECs) after extraction using phosphoric acid. (A) Frequency histogram data of particle diameter (μm) for 1000 defatted pollen grains and 1000 SECs. Optical micrographs of (B) defatted pollen grains and (C) SECs after acidolysis using phosphoric acid.

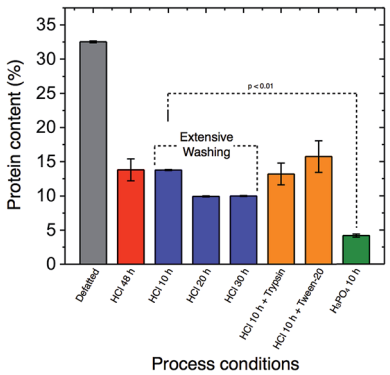


Fig. 5 Residual protein content in sunflower sporopollenin exine capsules (SECs) after different chemical processes, as measured with CHN elemental analysis. The protein content is a measure of pollen protein constituents in unprocessed and processed SECs and is determined by using the nitrogen percentage from elemental analysis with an assumed multiplication factor of 6.25 to convert nitrogen percentage into protein percentage. Data is presented as the average of triplicate measurements with standard deviation ($n = 3$).

similar structural ornamentation as unprocessed sunflower pollen grains.

Finally, we investigated the amount of residual protein present in sunflower SECs obtained using different processing schemes by performing CHN elemental analysis before and after SEC extraction. It is well known that proteinaceous nitrogen is one of the major components of plant materials[16] and defatted sunflower pollen contains ~32 wt% of proteins (Fig. 5). The acidolysis process using HCl for 48 h yielded ~14 wt% residual protein. The residual protein content remained nearly unchanged between ~10 wt% and ~14 wt% with varying duration of HCl acidolysis treatment and extensive washing. Further, in an attempt to eliminate proteinaceous material from SECs, trypsin or Tween-20 were used as additional washing solutions. Our CHN elemental analysis indicates that there was no improvement in the removal of proteinaceous nitrogen using trypsin or Tween-20. Strikingly, acidolysis using phosphoric acid produced sunflower SECs with significantly ($p < 0.01$) lower protein content (4 wt%) compared to SECs produced by acidolysis using HCl (see CHN data in Table S2†).

4. Conclusions

A process was developed in order to isolate intact, clean sunflower SECs without altering their native microstructure and surface ornamentation. Indeed, the improved process achieves the twin goals to preserve the SEC microstructure and remove a high fraction of pollen protein constituents. On the other hand, the data also supports that the SEC microstructure is damaged by alkaline lysis, which is a long established step in SEC extraction. This discovery underscores the potential significance of simplified processing schemes that not only reduce processing time but also improve the quality of the final product. By contrast, herein, we show that the unique, intrinsic microstructure of pollen is retained after removal of all pollen constituents by a simple acidolysis process using phosphoric acid. Our extraction process provides access to further explore the wide range of available natural pollen grains. To our knowledge, sunflower SECs produced with this optimized process offer a unique microstructure with a large inner cavity for diverse microencapsulation applications including drug delivery, cosmetics, and food technology.

Acknowledgements

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