DOI https://doi.org/10.1016/j.ejop.2012.07.002

Isolation of alveolar plates from *Coleps hirtus*

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Abstract

In the ciliate *Coleps hirtus*, the alveoli contain rigid alveolar plates that are almost unstudied so far. Neither the exact composition nor the genesis and function are known. A necessary step to study the alveolar plates is to isolate these structures in an adequate amount. Therefore, culture conditions of *C. hirtus* were optimized to obtain an axenic and dense long-time culture. The protocol we developed to isolate *C. hirtus* alveolar plates is presented and clean alveolar plates were documented via scanning electron microscopy. The described procedure delivers alveolar plates of very good structure and integrity with preserved filigree details in sufficient amount. They can be analyzed via a range of different material and biological characterizations. Since there are indications of a mineral phase within the alveolar plates, the presented results will allow to study *C. hirtus* alveolar plates also in the context of biomineralization.

Keywords: *Coleps hirtus;* Alveolar plates; Culture; Isolation; Alveolata

Introduction

Structure, composition and genesis of biological hard structures are relevant from a biological point of view. Furthermore, they are also often used as a source of inspiration for biomimetic processes and materials. Regarding biomaterials and biominerals in Protozoa, prior work mainly focused on the ultrastructure of these materials, while few studies also characterized some material properties (Faurét-Fremiet and Gauchery, 1957; Hausmann and Walz, 1979). Since diverse skeletal structures occur in the protozoan cells, it seems to be promising to study them from a material science point of view. Therefore, sufficiently large long-term cultures are required to study the principles of material formation and to isolate adequate amounts of skeletal structures.

The protozoan cell body is provided by intra- and extracellular skeletal elements that could be composed of organic and/or inorganic materials (Foissner et al., 1999). One example for cortex skeletal elements are the so called alveolar plates. Different types of these plates can be found within the alveoli, flattened vacuoles located beneath the plasma membrane that are a morphological character for the group Alveolata. It was supposed that the alveoli support large cell size by rigid external cell walls and that the alveolar plates function in stabilizing the cell (Cavalier-Smith, 1991; Hausmann and Hülsmann, 1996). Gould and co-workers presented a family of new proteins, alveolins, which might play a role as structural proteins generating rigidity (Gould et al., 2008).

The alveolar plates of the genus *Coleps* (Ciliophora, Prostomatea) are species-specific and of complex architecture (Chardez, 1976; Chen et al., 2010; Foissner et al., 1999; Kahl, 1930; Maupas, 1885; Noland, 1925). So far, functions as well as material characteristics are not known. Faurét-Fremiet did some cytochemical stainings and postulated that the ‘crustules’ are composed of calcium phosphocarbonates linked to a polysaccharide substrate (Fauré-Fremiet et al., 1968). To further analyse the alveolar plates, isolated clean alveolar plates are mandatory. To this end, we developed a protocol to isolate alveolar plates from *Coleps hirtus*. This species

occurs in fresh water habitats all over the world and feeds on other ciliates, flagellates, algae, bacteria and dendrites (Foissner et al., 1999; Maupas, 1885; Noland, 1925). *C. hirtu*s is able to live within wide limits of external conditions and it is found at all seasons of the year (Noland, 1925). As a matter of interest, some species of the genus occur in freshwater but others even in brackish water and marine. So far, laboratory cultures of four *Coleps spetai* clones were studied by Weisse and Rammer (2006) to investigate clonal differences; *Coleps* sp. and *C. hirtus* were studied regarding food selection (Klaveness, 1984; Madoni et al., 1990; Weisse and Rammer, 2006). These studies were taken as a basis for our aim to establish an axenic long-term *C. hirtus* culture that is suitable for alveolar plate isolation and characterization.

Material and Methods

Cultures

Wild type *Coleps hirtus* subspecies *hirtus* (O. F. Müller, 1786) Nitzsch, 1827 cells were collected 2009 from the lake Trentsee (Plön, Germany) and the species was determined using morphological characterizations described by Foissner and colleagues (Foissner et al., 1999). Individual cells were separated and axenic cultures were grown in Wright's cryptophytes (WC)-medium (pH 7.6-8) (Guillard and Lorenzen, 1972). Cultures for alveolar plate isolation were grown in the dark at 18-19 °C. *C. hirtus* was cultured either in Erlenmeyer or Fernbach flasks providing a large bottom area without shaking. Feeding was performed daily except for the weekends. As food source, *Cryptomonas* sp. (SAG 26.80, SAG culture collection Göttingen, Germany) was offered. *Cryptomonas* sp. was grown in Desmediacean medium ($MiEB_{12}$, SAG, http://epsag.uni-goettingen.de) at 20 °C with a photoperiod of 12 h/12 h. Before feeding, *Cryptomonas* sp. were washed by centrifugation at 4100 *g* for 4 min (Rotana 460 R, Hettich) and afterwards resuspended in WC medium. The amount of food was adjusted to the cell

density, i.e. approximately 1.1 x 10^6 *C. hirtus* cells were fed with approximately 11 x 10^6 cells of *Cryptomonas* sp.. The culture was regularly cleaned by using a 40 µm filter. *C. hirtus* was concentrated via oil test centrifugation at 230 *g* for 2 min (6-10, Sigma). If necessary, food was removed by collecting *C. hirtus* cells in a 10 µm filter. Both *C. hirtus* and *Cryptomonas* sp. cultures were treated under sterile conditions.

A THOMA chamber and a microscope (Axioskop, Zeiss) were used for counting *Cryptomonas* sp. cells that were pre-treated with KCl. *C. hirtus* cells were counted untreated using a stereo microscope (SZH10, Olympus). Growth rates (µ, d^{-1}) and doubling time (DT) of *C. hirtus* was calculated as follows (Madoni et al., 1990):

$$\mu\ (d^{-1}) = (\ln N_t - \ln N_0)/t\text{-}t_0)$$

$$DT\ (d) = \ln 2/\mu$$

where N_0 is number of ciliates at the beginning t_0 and N_t at the end (t) of one experiment. One experiment typically lasted five days with daily counting. Seven counting series were performed for standard culture conditions plus six series for conditions with constant culture volume.

Microscopy

Light microscopy (LM) as well as scanning electron microscopy (SEM) was performed to evaluate the isolation procedure. LM was applied after all isolation steps to control the condition of the alveolar plates and possible contaminations (Axiovert 200 M, Zeiss). After isolation, cleaning and storage of 4 weeks, SEM was applied on isolated alveolar plates. Therefore, acetone was used to transfer alveolar plates on a sample holder. Documentation was taken out

without any coating at 15 kV using a Hitachi TM-1000 electron microscope (Hitachi) with a backscattered electron detector.

Results

The subspecies *Coleps hirtus hirtus* was determined by morphological characters summarized by Foissner and colleagues (Foissner et al., 1999). In Figure 1 a single *C. hirtus* cell is shown by light microscopy. The cell is covered by alveolar plates and the whole armour consists of 6 plate rings each usually composed of 15-16 plates. Both anterior and posterior main plates have 4 windows while collateral plates have 2 windows (indicate in Fig. 1 a, b).

C. hirtus was successfully cultured for four years under laboratory conditions that were optimized for a later isolation and characterization of the alveolar plates. Results for *C. hirtus* culture are displayed in Table 1. *Cryptomonas* sp. was cultured as food source at a density of about 5.5 x 10^5 cells/ml. The average cell density of the *C. hirtus* culture was about 4500 cells/ml with a doubling time of about 7.3 d (standard culture). If the medium was exchanged in a way that the culture volume kept constant, a density of about 11,000-12,000 cells/ml was reached and the doubling time was increased to 4.47 d. About 18,000 cells/ml was the maximal amount of cells counted for one culture flask.

The isolation protocol that was developed for alveolar plates of *C. hirtus* is summarized in Figure 2. One week prior to isolation, feeding of the *C. hirtus* cultures was stopped. The culture was filtered through a 40-µm filter (Sefar Nitex, Sefar) and afterwards concentrated using conical oil vials for oil test centrifugation at 230 *g* for 2 min (6-10, Sigma). A pellet of *C. hirtus* was obtained for homogenization by additionally centrifugation within a 10 ml pointed test

glasses in a swing-out rotor (Rotofix II, Hettich). For all following steps the pellet was kept on ice. Homogenization was successful using 30 mM phosphate buffer pH 8.4 containing 1x EDTA-free protease inhibitor mix (Roche) within a glass homogenizer. The homogenate was transferred to a pointed test glass. After 40 min of sedimentation at 1 *g*, mainly connected alveolar plates sedimented while single alveolar plates and other cell compartments remained within the supernatant. The supernatant was removed for two more sedimentations at 1 *g* for 40 min. Sediments were collected separately and resuspended with cold acetone (Roth); another sedimentation at 1 *g* for 30 min was taken out in a clean test glass. Two more washing steps with acetone were performed afterwards in 1.5 ml test tubes while plates were sedimented via centrifugation at 1670 *g* for 1 min (MiniSpin, Eppendorf). Finally, the supernatant was removed and the remaining acetone was evaporated at 4 °C.

The development of a proper homogenization buffer was crucial, and a pH ≥ 8.4 was necessary for stabilizing the alveolar plates. EDTA containing buffers were not suitable. The plates stayed stable if they are carefully centrifuged, while they were damaged after Percoll gradient centrifugation. Alveolar plates are stable in acetone and can be stored dry at -18 °C for at least four weeks.

Isolation of alveolar plates was taken out for five litre of *C. hirtus* culture (containing about 22 x 10^6 cells) resulting in about 2.2 mg alveolar plates. Alveolar plates obtained from the first sedimentation are usually still connected (Fig. 1 c), while the second and third sedimentations provide more single alveolar plates (Fig. 1 d). Both parts were examined by scanning electron microscopy (SEM) revealing that the alveolar plates were intact and free of other cell compartments.

Discussion

We successfully investigated culture conditions suitable for alveolar plate isolation of *C. hirtus*. An axenic long-time culture was established. This was obtained by selecting WC-medium as culture medium and *Cryptomonas* sp. as food. Other food sources such as, e.g. yeast or animal tissue were tested but were not suitable for long-term axenic culture conditions. Cultures of *C. hirtus* were kept in the dark to avoid contamination with photosynthetic organisms. If necessary, *Cryptomonas* sp. can easily be removed by filtration due to its different cell size.

These long-term culture conditions resulted in doubling times of 4-7 d, which is longer than expected. Offering *Cryptomonas* sp., Klaveness obtained *Coleps* sp. cultures with doubling times (DT) of 2.47-1.12 d (growth rates (μ) 0.28-0.62 d^{-1}) while Madoni and co-workers fed different strains of *C. hirtus* with *Cryptomonas* sp. and documented a DT of 1.52 d (Klaveness, 1984; Madoni et al., 1990). These values were obtained for short culture periods (up to two weeks) and small volumes. Furthermore, Madoni and colleagues used sterile lake water that might support growth of the culture. Beside culture conditions and age of the culture, DT is influenced by the isolated *C. hirtus* strain.

The isolation of clean alveolar plates was successful and the yield of isolated alveolar plates was sufficient for future examination of the material properties. SEM investigations revealed that the alveolar plates were clean and free of contamination which is very important for material characterization of the plates. Beside an effective isolation of the plates, it was crucial to find conditions that stabilise the alveolar plates. Although they are thought to function as rigid elements (armour) to protect the cell against invertebrate predation (Foissner et al., 1999) and SEM freeze-etched studies suggested stable structures (Huttenlauch, 1985), the alveolar plates were sensitive to strong centrifugation, complexing agents and to low pH values. The fact that alveolar plates are sensitive to low pH values and complexing agents indicates that at least parts of the alveolar plates are mineralized. This was proposed by Maupas (1885) and Fauré-Fremiet (1944; 1948) but so far the alveolar plates were not examined using modern methods to confirm these results. Unique material properties of the alveolar plates in *C. hirtus*

would explain why other isolation protocols like for example for trichocysts (Glas-Albrecht and Plattner, 1990; Glas-Albrecht et al., 1992), *Paramecium* cortex (Stelly et al., 1991) or alveolar plates of *Euplotes* (Böhm and Hausmann, 1981; Hausmann and Kaiser, 1979) were not adaptable. Some of our observations further indicate a possible amorphous rather than a crystalline material.

Both, living and especially dividing *C. hirtus* cells as well as isolated alveolar plates are now available in a suitable amount to further study the genesis and the material properties of the alveolar plates. Since recently Foissner and colleagues also reported biomineralized silicon in a ciliate (Foissner et al., 2009), it will be very promising to further study biomaterials and biominerals in ciliates and other Protozoa.

Acknowledgements

This work was supported by the “Deutsche Forschungsgemeinschaft” (DFG PAK 410) and the COST Action TD0903, SEM pictures were taken at the Université de Bourgogne, UMR CNRS 5561 Biogéosciences in cooperation with Dr. Frédéric Marin.

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Figure legends

Figure 1. Alveolar plates of *C. hirtus*, a: light microscopy of *C. hirtus* when still alive, b: alveolar plates after homogenization (scales: 10 µm); c: SEM image of isolated connected alveolar plates (scale 30 µm), d: single alveolar plates (scale 10 µm)

Figure 2. Flow chart for *C. hirtus* alveolar plate isolation procedure

Table 1. Obtained cell densities and doubling times for culture of *C. hirtus*

Figure 1

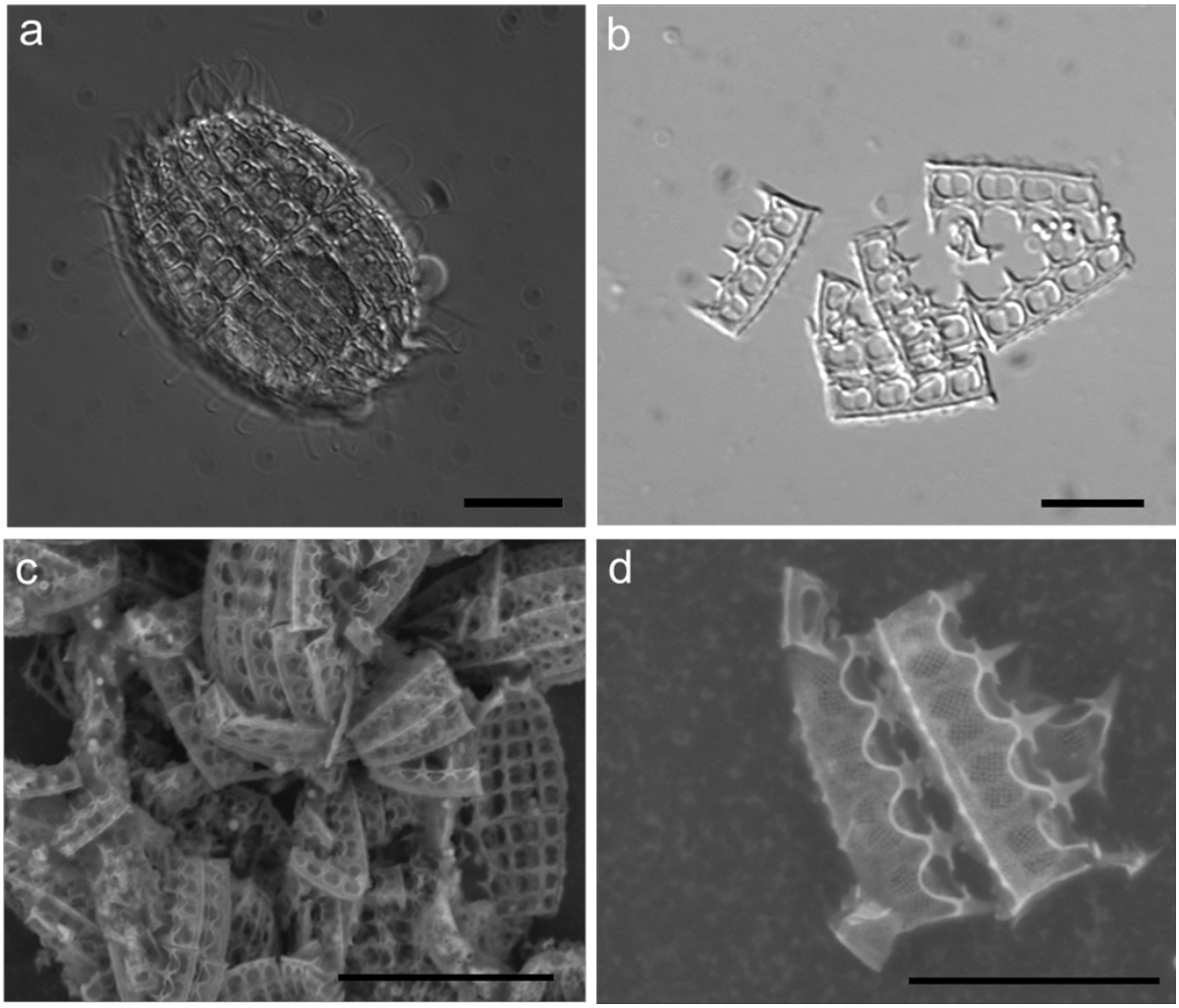

Figure 2

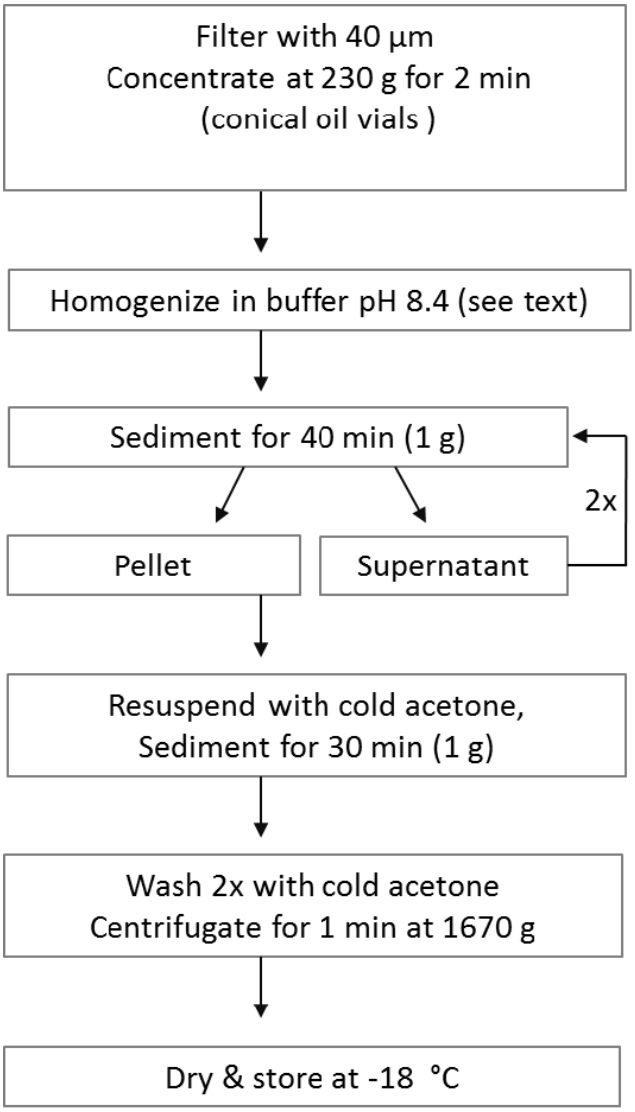
Filter with 40 µm
Concentrate at 230 g for 2 min
(conical oil vials)
Homogenize in buffer pH 8.4 (see text)
Sediment for 40 min (1 g)
2x
Pellet
Supernatant
Resuspend with cold acetone,
Sediment for 30 min (1 g)
Wash 2x with cold acetone
Centrifugate for 1 min at 1670 g
Dry & store at -18 °C

Table 1

Culture conditions	*Coleps hirtus* culture properties	
Standard culture	Cell density (mean value)	4,525 cells/ml
	Doubling time (mean value)	7.3 d
	(min; max)	(15.5 d; 2.6 d)
Constant volume culture	Cell density (mean value)	11,861 cells/ml
	Doubling time (mean value)	4.47 d
	(min; max)	(6.86 d; 3.25 d)