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The structural, compositional and mechanical features of the calcite shell of the barnacle *Tetraclita rufotincta*

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ABSTRACT

The microstructure and chemical composition of the calcite shell of the sea barnacle *Tetraclita rufotincta* (Pilsbry, 1916) were investigated using microscopic and analytical methods. The barnacle shell was separated mechanically into its three substructural units: outer, interior, and inner layers. The organic matrices of these structural parts were further separated into soluble and insoluble constituents and their characteristic functional groups were studied by FTIR. Investigation of the mechanical properties of the interior mass of the shell reveals remarkable viscoelastic behavior. In general, the mechanical behavior of the shell is a function of its geometry as well as of the material, of which it is constructed. In the case of *T. rufotincta*, as calcite is a brittle material, the elastic behavior of the shell is apparently related to its micro- and macroarchitecture. The latter enables the shell to fulfill its primary function which is to protect the organism from a hostile environment and enables its survival. Our detailed identification of the similarities and differences between the various structural components of the shell in regard to the composition and properties of the organic component will hopefully throw light on the role of organic matrices in biomineralization processes.

1. Introduction

The barnacle *Tetraclita rufotincta* (Cirripedia, crustacea) is abundant in the western Indian Ocean and off the coast of East Africa (Chan et al., 2009). Barnacles are crustaceans that are permanently cemented to the substrate by a multiprotein complex (Bourget, 1987; Mori et al., 2007). They inhabit shallow and tidal areas, mostly in the sublittoral zone (Doyle et al., 2007), and are highly adapted to a sessile mode of existence. The hard shell that surrounds the animal protects it against the physical and mechanical pressures of its habitat and enables it to survive periodic desiccation of the tidal zone. According to the official taxonomical hierarchy used in zoology, the name *T. rufotincta*, Pilsbry (1916) is the official name of the specie, even though originally Pilsbry had been classified the specie as *Tetraclita squamosa rufotincta* (*T. s. rufotincta*). However it seems that the status of *T. rufotincta* is still under discussion and the name *T. s. rufotincta* sometimes used for the specie (Achituv and Barnes, 1978; Chan et al., 2009; Lowenstam and Weiner, 1992). Further studies must be conducted on molecular analysis to compare the genetic differentiation, to further ascertains the taxonomic status of the barnacle.

Although the barnacle shell has great adaptive significance, few studies have focused on its microstructure. While the general structure and composition of the shell plates of some barnacle species have been investigated (Bourget, 1977; Lowenstam and Weiner; 1992; Rodriguez-Navarro et al., 2006), there has been no detailed study of the relationship between the shell's architecture and its mechanical properties, and little is known about the organic substances in the shell. Yet it is generally recognized that the organic component is critical for biomineralization processes: it controls the formation of crystals of specific morphology and orientation and outlines the design and properties of the shell (Aizenberg et al., 1996; Arias et al., 2004; Bezares et al., 2008; Nudelman et al., 2006).

This work focused on the range of substructural elements that have been observed in the shell of *T. rufotincta.* The crystalline microstructure of the mineralized shell was studied using FTIR, light microscopy and electron microscopy (SEM). Results relating to the organic matrix of the different layers of the shell plates were correlated with their observed structural features. Our analysis of the mechanical and structural properties of the shell shed some light on how a feature such as the shell's architecture fulfils its mechanical function. It is hoped that the similarities and

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differences, found between the various layers of the shell in regard to overall composition and properties of the organic component, will contribute to a better understanding of the significance of these organic matrices for biomineralization processes.

2. Materials and methods

2.1. Sample preparation

Specimens of *T. rufotincta* were collected in Eilat from the upper belt of the shallow seawater zones adjacent to the Interuniversity Institute for Marine Science (IUI) of the Red Sea, Eilat, Israel. The overall diameter of the shells included in this study was 4.5–5.0 cm, the maximal size which reaches matured barnacle (Achituv and Barnes, 1978). The animals were removed and the shells bleached in 2% sodium hypochlorite solution for 30 min. For evaluation of density, porosity, and mechanical properties, pieces measuring $10 \times 5 \times 5$ mm were cut from the honeycombed interior mass of the shell using a diamond saw, then ground with an increasingly fine sequence of sandpapers. For evaluation by FTIR, pieces were cut in the similar manner from all three structural layers then roughly sorted into three groups according to provenance.

2.2. Three-dimensional imaging

A three-dimensional model of the honeycombed internal mass was constructed from a series of 2D images, obtained using of a bifocal optical microscope coupled with a digital camera (Nikon) and a scanning electron microscope (JEOL, JSM-5610-LV). The images were analyzed using 2D/3D image-analyzing software (Image-Pro Plus; Media Cybernetics and ImageJ, NIH).

2.3. Light microscopy and scanning electron microscopy (SEM)

Shell sections were observed and photographed under crossed nicols in transmitted light. A Nikon SMZ1500 stereoscope equipped with Nikon DMSX digital camera was used to take microphotographs. Some specimens were etched after fractionation with HCl (0.5 N) for 1 min and then washed with distilled water and air dried. Organic matrices for SEM studies were obtained by decalcifying the shell fractions in EDTA (5% solution, 1 week duration). After decalcification, they were dehydrated in ethanol and air dried. For evaluation by SEM (JEOL, JSM-5610-LV) all samples were sputter coated with a 15 nm of gold.

2.4. Density and porosity

The bulk density of the honeycombed interior layer of the shell was measured in blocks measuring $10 \times 5 \times 5$ mm. The bulk density is defined as the ratio of the weight of the specimen to the total volume it occupies. The true density was measured using the buoyancy method. The total porosity was calculated as $\varphi_T = 100\left(1 - \frac{\rho_B}{\rho_G}\right)\%$, where φ_T is the total porosity, ρ_B the bulk density, and ρ_G the true density. The pore area and the distribution of pore area were measured from the digital microphotographs using NIH ImageJ software. Measurements were carried out on 57 specimens of the barnacle.

2.5. Mechanical studies

A compression test was performed using a universal loading machine (Instron, Model 4502, Instron Corp, Buckinghamshire, UK) equipped with a 10 kN load cell for a compression rate (cross-head speed) of 0.5 mm/min. Force was applied in the longitudinal and the transverse directions. Strain was calculated by dividing the deformation by the initial thickness of the specimen, and stress–strain diagrams were obtained. Ultimate compressive strength was defined as the maximum stress achieved during testing. The modulus of elasticity (Young's modulus) was calculated from the linear slope of the initial section of the stress–strain curve.

2.6. Organic matrix extraction and separation

The outer, interior and inner components of the barnacle shell were separated mechanically, placed in 3 kDa cut-off membranes, and decalcified with 5% EDTA (pH = 7.7) for 5–7 days. Following Yuehuei and Kylie (2003) (Yuehuei and Kylie, 2003) demineralization using EDTA is highly preferable for subsequent chemical analyses of the organic matrices. The traces of the EDTA in the organic matrices were removed by DDW exchanging, the efficiently of the rinsing was checked by comparison of FTIR spectrum of EDTA with the spectra of the organic matrices. No traces of EDTA have been detected. After decalcification, the entire extract was desalted by exchange with Milli-Q water, while the organic extract still fixed into the cut-off membranes. Then soluble organic matrix (SOM) was separated from insoluble organic matrix (IOM) using a centrifuge (10,000 rpm for 20 min), and all fractions were lyophilized. Total organic content was measured by weighing the total organic matrix after the decalcification procedure and vacuum drying.

2.7. FTIR analysis

Samples for FTIR analysis were prepared from the inner and outer layers and from the interior mass of the shell plates. Some samples were used undecalcified, while the rest were decalcified as described above. The samples were ground into a fine powder and mixed with KBr (1:1000 ratio) in an agate mortar. The FTIR spectra of all specimens, undecalcified and decalcified, were recorded at 4 cm^{-1} resolution with 64 scan on a Nicolet FTIR spectrophotometer 710, equipped with a broad range MCT detector. Spectra were recorded from 4000 to 400 cm^{-1}.

3. Results

3.1. Macro- and microstructure

The sea barnacles *T. rufotincta* are attached to a hard substrate in shallow water, they inhabit a tightly constrained location above and below sea water (Fig.1a). The shell of the sea barnacle *T. rufotincta* consists of an array of mineralized plates, known as mural or wall plates, which are joined together to form a truncated cone opened at the apex (Fig.1b–d). The interior mass of the plates have longitudinal canals, extended from the base to the apex. The thickness of the mature shell varies from 10 ± 2 mm at the base to 1 ± 0.5 mm at the top, the overall diameter reaches 4.5–5 cm.

The shell plates have a complex biocomposite structure, comprising a mineralized component combined with an organic matrix. Light microscope and SEM photographs of sections of the shell are shown in Fig.2. The shell consists of three distinct substructures: an outer layer (Fig.2a, d, g, j), a honeycombed interior mass (Fig.2b, e, h, k), and an inner layer (Fig.2c, f, i, l). Each of these layers has a distinct microstructure and mineral orientation. The outer layer has a rough surface and exhibits longitudinal striations extended from the base to the apex of the cone that are expressed on the macro as well as on the micro scale (Fig.2a, d). On the micro scale, transverse sections of the etching surface of the outer layer (Fig.2g) reveal an oriented conical structure formed by elongated crystals; the apparent size of the crystals lies in the range of 1–3 μm. If the etching surface is examined closely (Fig.2g), a fine net-