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Structural optimization and amorphous calcium phosphate mineralization in sensory setae of a terrestrial crustacean (Isopoda: Oniscidea)

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ABSTRACT

Terrestrial isopods possess large sensory setae on their walking legs. Increased fracture resistance of these elongated structures is of crucial importance, making the exoskeleton forming the setae an interesting durable material that may inspire biomimetic designs. We studied the cuticle of the sensory setae with analytical electron microscopy in order to gain detailed insights into its structure and composition at the nanometer scale and identify features that increase the fracture resistance of these minute skeletal elements. The setae are stiff structures formed by mineralized cuticle that are connected to the leg exoskeleton by a non-mineralized joint membrane. Our results demonstrate that different layers of the setal cuticle display contrasting organizations of the chitin-protein fibers and mineral particles. While in the externally positioned exocuticle organic fibers shift their orientation helicoidally in sequential layers, the fibers are aligned axially in the internally positioned endocuticle. In the setal cuticle, layers of structurally anisotropic cuticle likely providing strength in the axial direction are combined with layers of isotropic cuticle which may allow the setae to better resist perpendicular loading. They are further strengthened with amorphous calcium phosphate, a highly fracture resistant mineral rarely observed in invertebrate skeletons.

1. Introduction

Arthropods are a diverse animal group that forms exoskeletons in which different skeletal elements are evolutionary optimized to perform different functions, ranging from protection and movement to the manipulation and sensing of the environment (Moussian, 2013). The study of specialized skeletal elements, particularly ones subjected to heavy loading, can inspire biomimetic designs and high-performing materials with fine-tuned mechanical properties (Meyers et al., 2008; Paris et al., 2013; Wegst et al., 2015).

Crustaceans are an arthropod group with differently mineralized exoskeletons (Luquet, 2012). Furthermore, they exercise great control over the formation of various minerals, including calcite, apatite, and silica, which they incorporate in their cuticle (Bentov et al., 2012; Hild et al., 2008; Matsko et al., 2011; Miller et al., 1990). The cuticle forming the crustacean exoskeleton is a biological composite material, consisting of an organic phase of chitin-protein fibers and a mineral phase, predominantly consisting of calcium carbonate (Nikolov et al., 2011; Raabe et al., 2005). The cuticle is generally divided into three distinct layers; the mostly proteinaceous epicuticle, which is the thin outermost layer, is generally not strongly mineralized. The two inner layers, the exocuticle and the more proximal endocuticle, contain chitin-protein fibers and are usually mineralized with calcite and amorphous calcium carbonate (Hild et al., 2008, 2009; Neues et al., 2007). In the cuticle forming the dorsal plates (tergites) of crustacean exoskeletons, the chitin-protein fibers are oriented in a twisted plywood-like arrangement known as the Bouligand pattern. In this organization, the fibers form horizontal sheets of roughly parallel fibers, with the direction of the fibers shifting by a certain angle relative in sequential sheets (Bouligand, 1972; Nikolov et al., 2011). The local structure and composition of the exoskeleton can be fine-tuned at the micrometer or nanometer scale both by modifying the mineral composition and the organic fiber orientation, optimizing the mechanical performance of the material, a quality that may be worth mimicking in synthetic composites (Nikolov et al., 2011; Raabe et al., 2005).

Terrestrial isopods or woodlice achieved the greatest degree of terrestrialization among crustaceans (Hornung, 2011). This makes them important models for the study of exoskeletal mechanics, as the terrestrial environment imposes different mechanical demands on the skeleton of terrestrial isopods as compared to their aquatic relatives.

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Furthermore, the study of woodlice may elucidate the differences and similarities in mechanical solutions between them and insects, another successful terrestrial arthropod group.

Arthropods utilize sensory setae to communicate with their environment by detecting chemical and mechanical stimuli, temperature, and humidity (Keil, 1997). Often, mechanoreceptors of arthropods are cuticular setae capable of deflecting and translating their deformations to sensory cells in the epidermis (Keil and Steinbrecht, 1984). Large, spine-like sensory setae on the pereopods are a conspicuous feature of woodlice. These setae are likely mechanoreceptors, as indicated by electrophysiological measurements of responses to mechanical stimulation of setae on pereopods of the beach slater *Ligia oceanica* (Alexander, 1971). Furthermore, electrophysiological measurements on morphologically similar setae on the antennae of the cave woodlice *Titanethes albus* demonstrated that they respond to mechanical stimulation (Crouau, 1995).

As the sensory setae are exposed, elongated structures that likely come in contact with the substrate on which the animal moves they must necessarily be highly fracture resistant in order to maintain their functionality. This is potentially applicable to biomimetic design of minute machine elements, as the setae are only a few tens of micrometers in size, yet superbly withstand repetitive loading. Their structural and compositional features could thus be mimicked in the design of small machine elements at comparable size scales. The purpose of our study was to reveal the orientation of organic fibers and the mineral composition of the sensory setae on the pereopods of the woodlouse *Porcellio scaber* in order to identify specific structural features that enable them to resist fracture. The need for both stiffness and fracture resistance requires a compromise in the mechanical properties of such setae, making them an interesting model for the study of evolutionary optimization of such elements with potential application in biomimetic materials design.

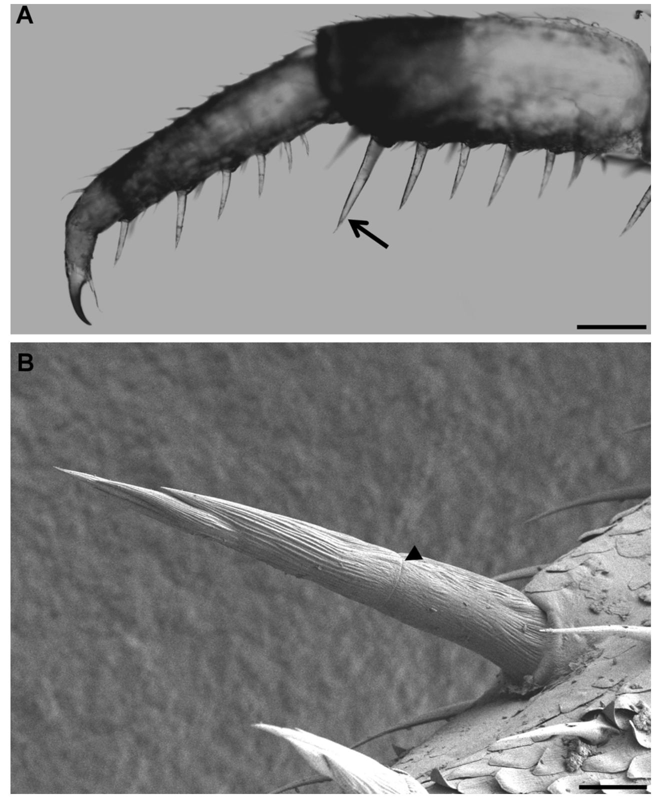

Fig. 1. Sensory setae on pereopods. A: Light micrograph of a *Porcellio scaber* pereopod showing sensory setae (arrow). Scale bar: 200 µm. B: Scanning electron micrograph using secondary electrons showing a sensory seta. The seta has an articulated base and ends in 5 points. Its surface forms longitudinal ridges and it possesses an annulus (arrowhead). Scale bar: 20 µm.

2. Material and methods

2.1. Animals

Adult *Porcellio scaber* individuals were maintained in a laboratory culture at room temperature and provided with leaf litter as food. For the analysis of setae on pereopods, individuals were isolated and maintained in glass petri dishes on wet filter paper for a week before the fixation. Animals that had not molted during the previous week and did not possess sternal $CaCO_3$ deposits that indicate the premolt stage (Zidar et al., 1998) were used for further analyses.

2.2. Whole-mount observations

For observation of the response of setae to loading, fresh pereopods were attached to glass slides with cyanoacrylate glue (Henkel) in petri dishes. Wet filter paper was added to the petri dishes to prevent desiccation during curing of the glue. In order to observe their behavior upon loading, the setae were experimentally deflected with an entomological pin. Images were acquired with a BX51 light microscope (Olympus) equipped with a DP70 digital camera (Olympus).

2.3. Scanning electron microscopy (SEM)

For SEM, pereopods were fixed in absolute ethanol, immersed in hexamethyldisilazane (HMDS) and air dried. Dry pereopods were attached to metal holders with silver paint (SPI) and sputter-coated with Pt for structural observation. Images were recorded with a JSM-7500 F field emission SEM (JEOL Co. Ltd.) operated at 5 kV. Low-magnification elemental mapping of bulk specimens was done on block faces of methanol-fixed samples embedded in Spurr's resin (SPI) and microtome-polished using a diamond knife. Elemental mapping was performed with a DSM 982 Gemini SEM (Zeiss) equipped with an energy dispersive X-ray detector and operated at 5 kV.

2.4. Conventional transmission electron microscopy (TEM) of decalcified samples

For TEM, pereopods were dissected and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.3). The fixative was then washed and pereopods were decalcified with 10% w/v aqueous EDTA. Tissues were post-fixed with 1% aqueous OsO_4, dehydrated in an ascending concentration series of ethanol and embedded in Spurr's resin (SPI). Sections were collected on copper grids and contrasted with uranyl acetate and lead citrate. Images were obtained with a Phillips CM 100 transmission electron microscope.

2.5. Analytical scanning transmission electron microscopy (STEM)

For the analysis of the mineral components of the setal cuticle, dissected pereopods were immediately dehydrated in methanol and embedded in Spurr's resin. Sections were cut with a diamond knife (Diatome) using a Leica EM UC6 ultramicrotome (Leica Microsystems) and collected on support copper grids covered with lacey carbon. High-angle annular dark-field (HAADF) -STEM images and energy dispersive X-ray (EDX) spectra were obtained at 200 kV with an advanced TEM/STEM (JEOL ARM200 F, JEOL Co. Ltd) microscope equipped with a cold field-emission gun and a DCOR probe Cs-corrector (CEOS Co. Ltd.). EDX spectra were obtained by using a 100 mm^2 JEOL Centurio SDD-EDX detector and a Thermo Noran System 7 EDX system (Thermo Fischer Scientific Inc.). Quantitative EDX analysis was performed using experimentally determined k-factors measured from standard hydroxyapatite (Ca phosphate) specimen under the same experimental conditions as for all other measured samples. The Zeiss SESAM microscope,