Journal of Structural Biology 174 (2011) 512–526

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Ultrastructure and mineral distribution in the tergite cuticle of the beach isopod *Tylos europaeus* Arcangeli, 1938

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ARTICLE INFO

Article history:
Received 23 December 2010
Received in revised form 7 March 2011
Accepted 8 March 2011
Available online 15 March 2011

Keywords:
Amorphous calcium carbonate
Biomineralisation
Calcite
Confocal Raman microscopy
Crustacea
Cuticle
Isopoda

ABSTRACT

The crustacean cuticle is a hierarchically organised material composed of an organic matrix and mineral. It is subdivided into skeletal elements whose physical properties are adapted to their function and the eco-physiological strains of the animal. Using a variety of ultrastructural and analytical techniques we studied the organisation of the tergite cuticle of the sand burrowing beach isopod *Tylos europaeus*. The surface of the tergites bear epicuticular scales, sensilla and micro-tubercles. A distal layer of the exocuticle is characterised by a low density of organic fibres and the presence of magnesium-calcite. Surprisingly, the mineral forms regions containing polyhedral structures alternating with smooth areas. Between sub-domains within the distal exocuticle calcite varies in its crystallographic orientation. Proximal layers of the exocuticle and the endocuticle are devoid of calcite and the mineral occurs in the form of amorphous calcium carbonate (ACC). Using thin sections of mineralised cuticle we describe for the first time that ACC forms tubes around single protein–chitin fibrils.

1. Introduction

The cuticle of Crustacea is a multifunctional exoskeleton that provides support and sites for muscle attachment. It protects the animal from mechanical stress and environmental hazards like desiccation and predation, and bears structures to receive and conduct sensory information. For most crustaceans studied so far, the cuticle consists of a hierarchical organised organic matrix composed of proteins and chitin, and a mineral phase consisting mainly of Mg-calcite, amorphous calcium carbonate (ACC) and amorphous calcium phosphate (ACP). Because of its outstanding mechanical properties, and its high potential in biomimetic research and technical applications the crustacean cuticle has attracted increasing interest.

In general, the organic phase of the arthropod cuticle comprises about seven hierarchical levels (Nikolav et al., 2011; Vincent, 2002). On the molecular level, antiparallel oriented chitin molecules (level 1) build α-chitin crystals (level 2) (Carlström, 1957). These crystals assemble to long, thin fibrils that are coated with proteins (level 3) in a helical manner (Blackwell and Weih, 1980). Fibres are built from bundles of fibrils (level 4). The fibres are arranged parallel to each other forming planes (level 5). These planes are stacked and the direction of the fibres in successive planes is twisted in a helicoidal manner. Thereby, the fibres form a so-called twisted plywood structure (level 6) (Bouligand, 1972). The distance between planes in which fibre orientation is twisted by about 180° is called stacking height. This distance becomes apparent in the layered appearance of sections of the cuticle when the cutting plane was slightly oblique to the surface (Giraud-Guille, 1984a; Raabe et al., 2005). The stacks built at least three main layers (level 7), called from distal to proximal the exocuticle, endocuticle and membranous layer. In crustaceans the former two are mineralised. In addition, the cuticle has an outermost epicuticle that contains no chitin and forms surface structures like scales and epicuticular bristles.

The crustacean cuticle is a composite material with high functional versatility. Its properties depend on the function of the skeletal element considered, and on ecological and behavioural differences between species. Potentially each of the seven hierarchical levels provides one or more degrees of freedom to alter the physical properties of the cuticle. Thus, in order to understand the structure–function relationship in crustacean cuticle, it is of interest to study the ultrastructure of skeletal elements of a variety of species with different behavioural characteristics and different habitats. Isopods are a large group of crustaceans of relatively uniform anatomy. Nevertheless, they inhabit a wide range of marine and terrestrial biotopes that require specific behavioural adaptations. Therefore, isopods are good models to study the relations between the architecture of exoskeleton, environmental strains and behaviour.

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doi:10.1016/j.jsb.2011.03.005

In addition to the organic phase, the type and distribution of the mineral phase as well as the concentration of magnesium and of phosphate influence the physical properties of the cuticle. A recent study on the tergite cuticle of six terrestrial and four marine isopod species has shown that the relative amounts of the amorphous and the crystalline phase varies between species, and suggested that the ratio depends on the function of the cuticle and the habitat of the animal (Neues et al., 2007). For the terrestrial isopods *Porcellio scaber* and *Armadillidium vulgare* (Crinochaeta) and for the cave dwelling isopod *Titanetes albus* (Trichonscidae) the distribution of calcite and ACC has been investigated (Hild et al., 2008, 2009). In the *Crinochaeta* species the calcium carbonate phase within the exocuticle is mainly calcite with little ACC, whereas the mineral phase of the endocuticle is amorphous and devoid of calcite. In *Titanetes* the distribution is somewhat different. Probably due to its troglobitic life, calcite is allocated to the distal region of the exocuticle only, while the proximal part of the exocuticle and the endocuticle contains ACC. At the nano-scale the spatial organisation of the mineral phase follows that of the organic chitin–protein fibres. In the crinochaet isopods 20 nm thick granules align to the fibres in the form of calcite or ACC in the exo- and endocuticle, respectively (Hild et al., 2008). A similar alignment of calcite granules have been observed in the decapod Crustacea *Carcinus maenas* and *Menippe mercenaria* (Roer and Dillaman, 1984), suggesting that the alignment of mineral granules to chitin protein fibres may be a general characteristic of mineralised cuticle architecture in crustaceans.

To further investigate the relation between the structure of the organic matrix, the distribution of the mineral phase and the behaviour and habitat of crustaceans, we investigate the tergite cuticle of Tylidae, a sister-group of all other terrestrial isopods (Oniscidea) except the Ligiidae (Erhard, 1997). The taxon comprises the monotypic genus *Helleria* that lives in forests and the genus *Tylos* that includes about 20 species that all live on sandy beaches where they burrow into the substrate (Schmalfuss and Vergara, 2000). They can roll up to a sphere to protect their soft ventral side against predators (Schmalfuss, 1984). During the night the beach dwelling species feed on algae and wrack washed ashore. Here we report our results on *Tylos europaeus* (Tylidae) that lives along European Atlantic coasts south of Bretagne (France), and along the coasts of the Mediterranean and Black Sea.

We used field emission scanning electron microscopy (FE-SEM) and low voltage scanning electron microscopy (STEM) for the structural analysis of the tergite cuticle. X-ray microprobe analysis (EPMA) and scanning confocal Raman spectroscopic imaging (SCμ-RSI) was used to map the elements, and the distribution of calcite, ACC and organic material, respectively. For a quantitative analysis of calcite, ACC and organic material we used a combination of X-ray diffractometry (XRD), thermogravimetry (TG) and atomic absorption spectroscopy (AAS). The results show that in the major distal part of the exocuticle the mineral phase has a radial growth pattern of calcite granules forming unusual polyhedral structures that alternate with smooth regions. Interestingly, this layer contains sub-domains of varying crystal orientation that were not observed in other Crustacea including isopods. We propose that these features are a consequence of the very low density of organic fibres within the distal exocuticle. Furthermore, we show that within proximal layers of the cuticle ACC forms tubes around single protein–chitin fibrils.

2. Materials and methods

2.1. Animals

T. europaeus Arcangeli, 1938, were collected from a beach of the estuarine system in Praia da Barra near Aveiro (Portugal). Animals were kept in a terrarium filled with sand. One side of the terrarium had small holes in the bottom. This side was placed into a seawater reservoir to wet the sand and to allow for exchange of ammonia. *Ulva lactuca* served as food. In this study we used intermoult animals only that were identified by the lack of sternal deposits and, during dissection, by the lack of an ecdysial gap. We used the tergites 2–7 for analysis. Some tergites were stored at −20 °C after treatment with 100% methanol and air-drying until further use.

2.2. Low voltage scanning transmission electron microscopy (STEM)

Additionally to frozen tergites pre-treated in methanol as described before, also tergites freshly removed were used. Frozen and freshly prepared tergites were decalcified and the organic matrix was fixed simultaneously by incubation in a solution containing 0.5 M EDTA, 2.5% glutaraldehyde, 2% paraformaldehyde and 0.25 mol L^{-1} HEPES, pH 7.8 for approximately 4 days at 4 °C. Then the tergites where washed three times in bidistilled H_2O for 10 min and postfixed for 1 h in a solution containing 1% OsO_4 and 0.8% $K_4[Fe(CN)_6]$. Tergites where washed again three times in H_2O for 10 min, dehydrated in a graded series of isopropanol, block-contrasted in an aqueous solution of 0.5% uranyl acetate, washed three times in isopropanol and two times in acetone for approximately 4 min each. Then the tergites were embedded in EPON resin. Sagittal ultrathin sections (25–70 nm) of the embedded tergites were cut using an Ultracut ultramicrotome (Leica, Austria). The sections where placed on 300 mesh copper grids or carbon coated formvar films on 1 mm hole grids, and stained with 2% uranyl acetate and 0.3% lead citrate and coated with a carbon layer of 4.5–7.5 nm using a BAF 300 freeze etch device (Balzers, Liechtenstein).

Sagittal ultrathin sections of mineralised tergites were cut dry using an ultra 45° diamond knife (Diatome, Switzerland). The sections were placed on carbon coated formvar films on 300 mesh grids. Darkfield STEM was performed using a field emission scanning electron microscope (FE-SEM) S-5200 (Hitachi) at an acceleration voltage of 30 kV and an emission current of 20 μA.

2.3. Scanning electron microscopy (SEM)

Tergites were prepared in methanol to preserve the mineral phase as described previously (Hild et al., 2008). Tergites were either cleaved in the sagittal plane or polished. To obtain polished surfaces, pieces of tergites where first glued on aluminium holders. A sagittal plane was cut using glass knives and an Ultracut ultramicrotome (Leica, Austria). Then a 45° diamond knife (Diatome, Switzerland) was advanced into the cuticle successively by 95, 70, 45, 20, 10 and 5 nm for at least 15 times each (Fabritius et al., 2005). Etched surfaces where obtained by incubating polished surfaces of the tergites for 60 s in an aqueous MOPS buffered solution (10 mmol L^{-1}, pH 6.5), and washing three times for 10 min in isopropanol to remove the aqueous solution. After critical point drying (Bal-Tec CPD 030) samples where rotary shadowed with 3.5–4 nm platinum at an angle of 45° using a BAF 300 (Balzers, Liechtenstein). FE-SEM (Hitachi, S-5200) was performed at an acceleration voltage of 4 kV and an emission current of 10 μA. For the analysis of tergite surface structures some samples were sputter coated with gold/palladium (Balzer MED 010) and analysed in a DSM 962 SEM (Zeiss, Germany) at an acceleration voltage of 20 kV.

2.4. Electron microprobe analysis

Electron microprobe analysis (EPMA) of sagittal surfaces polished as described above was performed with a S-5200 (Hitachi) FE-SEM equipped with a Phoenix (EDAX) X-ray-detector system and using GENESIS software. The microscope was set to 20 kV