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Ordering of protein and water molecules at their interfaces with chitin nano-crystals

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ABSTRACT

Synchrotron X-ray diffraction was applied to study the structure of biogenic α-chitin crystals composing the tendon of the spider *Cupiennius salei*. Measurements were carried out on pristine chitin crystals stabilized by proteins and water, as well as after their deproteinization and dehydration. We found substantial shifts (up to $\Delta q/q = 9\%$ in the wave vector in q-space) in the (020) diffraction peak position between intact and purified chitin samples. However, chitin lattice parameters extracted from the set of reflections (*hkl*), which did not contain the (020)-reflection, showed no systematic variation between the pristine and the processed samples. The observed shifts in the (020) peak position are discussed in terms of the ordering-induced modulation of the protein and water electron density near the surface of the ultra-thin chitin fibrils due to strong protein/chitin and water/chitin interactions. The extracted modulation periods can be used as a quantitative parameter characterizing the interaction length.

1. Introduction

Organisms produce a wide range of intricately structured biocomposites exhibiting hierarchical architectures at multiple length scales, from nanometers to millimeters. Vertebrate bones and teeth or mollusk shells are familiar examples, which are composites of biopolymers and minerals (Weiner and Wagner, 1998; Lowenstam and Weiner, 1989; Mann, 2001). On the other hand, plant materials such as wood are non-mineralized load-bearing biological composites comprised of polysaccharide fibers (cellulose) embedded in an amorphous polysaccharide (hemicellulose) and a complex aromatic alcohol polymer (lignin) matrix (Fengel and Wegener, 1984). Similar to wood, the spider cuticle, which is the subject of the current work, is a non-mineralized biomaterial built of semi-crystalline polysaccharide fibers (chitin) embedded into a hydrated protein matrix (Barth, 1973).

The interactions between the load-bearing components (polymeric fibers or minerals) and the (organic) matrix are of great importance to both the formation mechanisms and the mechanical performance of composite biological materials (Berman et al., 1988, 1990; Pokroy et al., 2004; Zolotoyabko and Pokroy, 2007). Importantly, the interaction of the organic molecules with specific atomic planes within biogenic calcium carbonate has been established (Berman et al., 1990; Pokroy et al., 2006a,b) and shown to induce anisotropic lattice distortions in the mineral. Distortions reach a maximum of about 0.2% along the c-axis in both biogenic aragonite and calcite and disappear upon mild annealing at 200–250 °C, which selectively destroys the organic matrix (Pokroy et al., 2004, 2006a,b).

The interactions between the proteins and the load-bearing chitin crystals in various arthropod cuticles have been studied using X-ray diffraction, biochemical methods, and molecular modeling. Early reports (Fraenkel and Rudall, 1947; Rudall, 1963) outlined the agreement between repeating distances along the c-axis in the orthorhombic chitin crystal structure (extracted from X-ray diffraction measurements) and the expected repeats along β-strands in pleated sheet motifs in specific matrix proteins, which would allow for multiple interactions between chitin and the proteins (Neville, 1993). Atkins (1985) identified another repeating distance of 0.475 nm, which is the spacing between chitin chains along the a-axis within the (010)-planes and also the distance between strands in a pleated protein β-sheet structure.

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In the arthropod cuticle, each chitin crystal is coated with globular proteins that give rise to additional features in the diffraction pattern of the cuticle, which is otherwise dominated by the diffraction from the chitin crystals. Blackwell and Weih (1980) showed the presence of axial layer-lines in the 2D X-ray diffraction pattern of the intact ovipositor cuticle of the wasp *Megarhyssa*, corresponding to a protein repeat of 3.06 nm along the chitin fibril and suggested that the protein consists of sub-units arranged in a 6_1-helix around the crystalline chitin core. It was also deduced that the protein/chitin interaction in the ovipositor of the wasp *Megarhyssa* takes place on the (0 1 0) chitin plane, presumably by multiple hydrogen bonds (Atkins, 1985; Blackwell and Weih, 1980; Vincent and Wegst, 2004; Neville, 1967).

Biochemical studies of proteins from a wide variety of arthropod cuticles identified a conserved chitin-binding sequence, the so-called R&R consensus sequence (Rebers and Willis, 2001). Using homology modeling, it was suggested that the preferred secondary structure of chitin binding domain in cuticular proteins, the so-called R&R consensus sequence, is an antiparallel β-sheet structure in a half β-barrel structure. In this configuration aromatic residues face the internal β-barrel surface and are expected to interact with the chitin crystals (Suetake et al., 2000; Iconomidou et al., 2005).

In this article, we shed additional light on the protein/chitin and water/chitin interactions, by studying X-ray diffraction profiles from biogenic chitin in its intact, deproteinized, hydrated and dried state. We model the protein- and water-induced modulation of the diffraction profiles, and track the apparent changes in the lattice parameter, *b*, upon dehydration and deproteinization. The investigated chitin crystals are the building blocks of the arthropod cuticle, specifically, the tarsal tendons of the wandering spider *Cupiennius salei*.

2. Materials and methods

2.1. Samples

Adult specimens of the Central American wandering spider *C. salei* (Barth, 2002) were obtained from the breeding stock at the Department of Neurobiology of the University of Vienna. The spiders were transported in dry ice and stored at −20 °C for 3 months at maximum. Tendons were pulled from the metatarsus segment of the leg and mechanically cleaned from muscle residues. The dorsal and the ventral tendons were indiscriminately used.

2.2. Partial and full deproteinization by basic treatment and bleaching

For partial deproteinization, tendons were immersed in 15 mL of 40% w/w KOH aqueous solution and heated at 70 °C under continuous stirring for 30 min. The samples were then thoroughly washed with distilled water and air-dried. The level of deproteinization was monitored by amino acid analysis (data not shown). Pure chitin – full deproteinization – was obtained by immersing tibiae cuticle samples in 0.3% NaClO solution buffered at pH 4.9 in 0.1 N acetate buffer solution at 70 °C for 3 h after an initial partial deproteinization treatment with KOH as described above.

2.3. Wide-angle X-ray scattering (WAXS)

Tendon samples attached to a silicon frame were mounted vertically in a custom-made humidity-controlled chamber and measured at a dedicated SAXS/WAXS station of the μ-Spot beam line at the synchrotron BESSY II (Helmholtz Center, Berlin, Germany). An X-ray energy of E_x = 15 keV (λ = 0.0826 nm) was chosen from the synchrotron radiation spectrum by a multilayered monochromator (bandwidth $\Delta E_x/E_x \approx 0.01$). The incident X-ray beam was focused on the sample by a toroidal mirror, and the final beam size was defined by a pinhole of 100 μm diameter placed in front of the sample. WAXS data were collected using a large-area 2D detector (MarMosaic 225, Mar USA Evanston, USA), situated approximately 300 mm behind the sample. The sample-to-detector distance was calibrated by measuring the diffraction from quartz standard.

Tendon and tibiae samples were measured at three different positions along the sample. For converting the 2D diffraction data into 1D profiles (as a function of the scattering vector, *q*), Matlab 8.6 software and the in-house SAXS/WAXS analysis software DPDAK (https://dpdak.desy.de/index.php/Hauptseite) were used. Selected diffraction profiles were fitted to pseudo-Voigt functions with a linear background using OriginPro9.1. For fitting peak positions, the scattering vector *q* was converted to *d*-spacing by applying Bragg's law ($d = 2\pi/q$). The extracted lattice parameters were determined using the peak positions of the (0 0 4), (0 4 2), (0 2 1), (1 1 0) and (0 1 3) reflections and averaged over the relevant measurement set.

2.4. Humidity control

A custom-made chamber was used to control temperature and humidity during X-ray measurements. The temperature was controlled using a thermostat (HUBER Ministat). The relative humidity (RH) was controlled using a WETSYS humidity generator (SETARAM) and was monitored by an EK-H4 Evaluation Kit (Sensirion) set up with a SHT75 capacitive sensor placed a few millimeters away from the sample. The accuracy of the RH and temperature readings was ±2% and ±0.5 °C, respectively. Humidity was set to a constant RH of 10% for "dry" samples and 90% for "wet" samples. All samples were equilibrated for one hour prior to diffraction measurements.

2.5. Raman spectroscopy

Spectra were acquired with a confocal Raman microscope (CRM200, Witec, Germany) equipped with a piezo scanner (P-500, Physik Instrumente, Karlsruhe, Germany). A linearly polarized near-infrared diode-laser (Toptica Photonics AG, Graefelfing, Germany) was used in combination with a Nikon 20× microscope lens. The spectra were acquired using an air-cooled CCD (PI-MAX, Princeton Instruments Inc., Trenton, NJ, USA) behind a grating (300 g/mm) spectrograph. The overall Raman intensity was maximized by adjusting the laser focus by means of an integrated light-microscope. The ScanCtrl Spectroscopy Plus software (Witec, Germany) was used for spectra acquisition (three hardware accumulations, ten software accumulations, 1 s integration time) and WiTec Project Plus was used for data processing.

3. Results and discussion

The tarsal tendon is used by the spider to flex its tarsus (the last segment of the leg) and to move the pretarsus containing the claws (Speck and Barth, 1982). The tendon is built of tightly packed α-chitin fibrils surrounded by proteins, which are aligned along the chitin *c*-axis (Fig. 1). The atomic structure of pure α-chitin was the subject of several comprehensive studies (Blackwell and Weih, 1980; Minke and Blackwell, 1978; Carlström, 1987; Sikorski et al., 2009). The space group is $P2_12_12_1$; and its orthorhombic unit cell has the following dimensions: a = 0.474 nm, b = 1.886 nm and c = 1.032 nm (Minke and Blackwell, 1978). The main motif of this structure comprises the adjacent polysaccharide chains running in antiparallel directions along the *c*-axis (crystallographic and fiber axis). The chains construct molec-