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Site-specific characterization of beetle horn shell with micromechanical bending test in focused ion beam system

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ABSTRACT

Biological materials are the result of years of evolution and possess a number of efficient features and structures. Researchers have investigated the possibility of designing biomedical structures that take advantage of these structural features. Insect shells, such as beetle shells, are among the most promising types of biological material for biomimetic development. However, due to their intricate geometries and small sizes, it is challenging to measure the mechanical properties of these microscale structures. In this study, we developed an in-situ testing platform for site-specific experiments in a focused ion beam (FIB) system. Multi-axis nano-manipulators and a micro-force sensor were utilized in the testing platform to allow better results in the sample preparation and data acquisition. The entire test protocol, consisting of locating sample, ion beam milling and micro-mechanical bending tests, can be carried out without sample transfer or reattachment. We used our newly devised test platform to evaluate the micromechanical properties and structural features of each separated layer of the beetle horn shell. The Young's modulus of both the exocuticle and endocuticle layers was measured. We carried out a bending test to characterize the layers mechanically. The exocuticle layer bent in a brick-like manner, while the endocuticle layer exhibited a crack blunting effect.

Statement of Significance

This paper proposed an in-situ manipulation/test method in focused ion beam for characterizing micromechanical properties of beetle horn shell. The challenge in precise and accurate fabrication for the samples with complex geometry was overcome by using nano-manipulators having multi-degree of freedom and a micro-gripper. With the aid of this specially designed test platform, bending tests were carried out on cantilever-shaped samples prepared by focused ion beam milling. Structural differences between exocuticle and endocuticle layers of beetle horn shell were explored and the results provided insight into the structural advantages of each biocomposite structure.

1. Introduction

Using or mimicking natural materials offers many benefits, as these materials have been optimized and varied over years of evolution. Cuticular structures of insects possess fascinating and diverse architecture range of micro-/nanoscale and the designs of the cuticles are the result of years of evolution, resulting in an optimized design [1]. Over the past decade, research into characterizing biological materials has flourished [2–9]. By understanding the useful features of biological structures, we can produce novel bioinspired structures. In terms of the mechanical properties of biological structures, the insect shell is one of the most interesting and frequently studied biocomposite materials. Insect shells possesses a high strength-to-weight ratio [10,11]. All types of insects

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form microstructured shells to protect themselves from external dangers. Especially, in the case of beetle, the beetle horn is used when male beetles fight during the mating season [12,13]. Thus, we expect the horn has good mechanical properties, including high stiffness and fracture toughness. The shell of the beetle horn has a multi-layered cuticle structure and possesses outstanding mechanical properties, as do other insect shells [14–17]. These desirable properties are derived from the unique internal features and composition of fibrous, composite-like structures. The insect cuticle consists mainly of a procuticle that provides shape and mechanical stability, as well as an epicuticle, which is a thin outer layer (thickness of 100–300-nm) [18,19]. The procuticle can be divided into two parts: the exocuticle and the endocuticle. The exocuticle has a thickness of 30–50 μm and the endocuticle, which is located under the exocuticle layer, has a thickness of 50–100 μm. Both layers consist of chitin fibers and proteins but frequently have different geometrical arrangements. The differences include the direction of lamination, the combination of chitin fibers, and the protein matrix [19]. Several researchers have investigated the mechanical properties of these biological materials. Hepburn et al. [15] examined the internal structure of beetle shells, including that of the procuticle, and measured the mechanical properties using tensile and torsion testing. Chen et al. [14,16,17] studied the macroscopic internal structure of beetle cuticles and proposed that they have a fiber-continuous panel-pillar structure, which has a high overall strength. In these studies, the procuticle was not separated into endocuticle and exocuticle layers, as the researchers were focused on the overall structure. To take full advantage of the biological material structures in biomimetic applications, it is essential to understand the structural characteristics and mechanical properties of each layer.

There have been significant advances in real-time imaging and sample preparation techniques over the past few decades. In-situ characterization/modification methods [20–27], for example, scanning electron microscopy (SEM), focused ion beam (FIB) and FIB-SEM dual beams, use nanomanipulators with mechanical, electrical, and/or thermal measurement probe attachments. The main advantage of this kind of in-situ characterization is that the measurement process can be operated under real-time observation; thus, it is highly reliable. There are various techniques to study mechanics on very small scales, such as tension, compression, bending, torsional and nanoindentation tests [24,28–30]. These methods are frequently combined with electron microscopy to take advantage of in-situ measurement techniques [25]. Many researchers have investigated the micromechanical properties of biological materials using FIB milling and micro-characterization systems [31,32]. FIB systems equipped with a variety of sensors have proven to be reliable with respect to their ability to resolve localized features at micro- and nanoscales. The most interesting feature of FIB technology is the localized sample preparation method, which uses a milling process. Using FIB milling, it is possible to fabricate a sample directly without the need for post-processing. The milling process can be used on almost any type of material and is not limited by the shape of the sample [33,34]. Orso et al. [4] demonstrated an in-situ test method for microscopic biological samples and measured the mechanical properties of a single beetle seta. Huber et al. [35] performed stress–strain measurements of gecko setae. However, it can be difficult to measure the mechanical properties of samples with intricate geometries, such as beetle shells with multi-layered fibrous structures. This is because the FIB sample preparation and handling process has limitations when extracting samples from specific locations and requires delicate, complicated procedures. Adineh et al. [36] characterized biomechanical structures in rat whiskers using FIB combined with atomic force microscopy (AFM). A micro-gripper system was used to overcome difficulties in controlling the sample whilst it is attached to the substrate. Some disadvantages, such as desiccation, arise from the use of a vacuum environment [4,37]. Whereas the nanoindentation technique, for characterizing hardness, elasticity or fracture, can be applied to a wetted sample or in a humidified chamber, measurements in this equipment are constrained by having to use a matrix to hold the material. This can introduce variability and errors. The FIB platform approach is superior in that it avoids the need for a matrix, but it is limited to dry samples unless a specialized cold stage is used that allows samples to be 'wet'. So, if used under full vacuum conditions the FIB platform should be seen as complementary to current approaches rather than a replacement technique.

In this study, an in-situ characterization method was developed using an FIB system to extract samples from a number of specific sites of the beetle horn cuticle; the method also allowed for micro-bend testing. The advantage of our set-up is that there is no need for the sample to be transferred from the sample preparation site to the testing site. Thus, this method reduces the risk of specimen misorientation and improves the accuracy of sample handling throughout the process.

2. Materials and methods

2.1. Composition of exocuticle and endocuticle of beetle horn shell

In this section, we outline the layered composite structure of the beetle horn shell and examine the microstructures of each layer. Material from a horned beetle (*Trypoxylus dichotomus*) was collected from *Tsukuba Botanical Garden, Amakubo, Tsukuba-shi, Ibaraki Pref., Japan* in August 2013. The horned beetle was collected using a banana trap, killed in 99.5% ethyl acetate vapor. The length of the beetles was 45–80 mm and length of the horns were 25–30 mm. Total 6 beetles were used for this experiment. After dissection, the horn was detached from the body and kept in 70% alcohol container for preservation [38,39], or dried for FIB process. As mentioned in the previous section, the beetle horn shell is composed of a number of layers, including the epicuticle, exocuticle, and endocuticle, as shown in Fig. 1(a). The outer layer of the epicuticle is not of interest in this research because it is known to be composed of waxes and contributes mostly to reducing evaporative water loss and protecting the insect from soaking [40–42]. Hence, it does not contribute to the mechanical properties of the shell. We focused on the mechanical properties of the procuticle layers. To distinguish the detailed structural differences between the exocuticle and endocuticle, microscopic images were taken using an FIB system (CORBRA-FIB; Orsay Physics, Fuveau, France). The sample preparation process and resulting microscopic images are shown in Fig. 1. The specimens were prepared for imaging by cutting off part of the beetle horn.

2.2. In-situ site-specific micromechanical test platform

As mentioned in Section 1, manipulating and characterizing microscale structures is a complicated process. When measuring biological materials, such as beetle shell, it can be difficult to determine an exact position due to the non-uniform shape and unpredictable composition of the material. This contrasts to the measurement of well-defined artificial materials. FIB has been used to prepare mechanical test specimens at the micro- and nano-scale in last two decades [36,43]. In our study, we used FIB to make the cantilevers of endocuticle and exocuticle to implement the micro-bending test. In the case of fiber bundle bending tests, however, extracting a single fiber bundle from the endocuticle layer is not possible using conventional FIB techniques. For this reason, we used a specialized gripping and detaching method to manipulate