Protoplasma (2001) 216: 71–74

PROTOPLASMA
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Printed in Austria

Cellulose in the house of the appendicularian *Oikopleura rufescens*

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Received June 10, 2000
Accepted October 2, 2000

Summary. By electron diffraction analysis, highly crystalline cellulose Iβ was found in the house (a special structure in which the tunicate lives) of the appendicularian *Oikopleura rufescens*. Cellulose microfibrils 20 nm in width were observed in a random array or highly organized with rectangular spacing of 2 to 10 μm in the house. The bundled cellulose microfibrils formed in the inlet filters, which are highly ordered meshwork structures. This paper provides the first account of the existence of cellulose in the house of an appendicularian. Our findings showed that the house and tunic are homologous tissues among the tunicates, and that the common ancestor of the tunicates (ascidians, thaliaceans, and appendicularians) already possessed cellulose-biosynthetic ability.

Keywords: Appendicularian; Appendicularian house; Cellulose; Electron diffraction; Tunicate.

Introduction

The tunicates (urochordates) are the only animals known to produce highly crystalline cellulose (Richmond 1991). The name "Tunicata" is derived from the unique integumentary tissue called the tunic, which contains the cellulose microfibrils. To date, cellulose I microfibrils have been found in almost all of the ascidians and thaliaceans (Belton et al. 1989, Daele et al. 1992, Okamoto et al. 1996, Hirose et al. 1999). Thus, the cellulosic composition of the tunic is considered to be a characteristic common to ascidians and thaliaceans in animals belonging to the subphylum Tunicata. The appendicularians are another group in the Tunicata. Although molecular phylogeny based on 18S rDNA sequences suggested that appendicularians share an ancestor with the other groups of tunicates (Wada and Satoh 1994, Wada 1998), they do not possess the tunic as an integumentary tissue. On the other hand, the appendicularians secrete a balloon-like, gelatinous structure called a "house" that acts as a feeding apparatus (see Flood and Deibel 1998). It is possible that the house corresponds to a kind of tunic in the appendicularians, but it is not yet clear whether the house contains cellulose. Therefore, it is necessary to clarify the existence of cellulose in the appendicularian house to understand the evolutionary pathway of cellulose biosynthesis in the tunicates.

The present study focused on the existence and characterization of cellulose in the appendicularian house. Our investigation provides a key to understanding whether the ability for cellulose synthesis is universal in the tunicates.

Material and methods

Sample collection and fixation

Appendicularians with their houses were collected in hand-held glass jars by skin divers at depths not exceeding 1 m off Maeda point, Okinawa, Japan. This method enabled us to obtain samples with minimal disturbance or mechanical damage to the fragile animals and their houses. After collection, the specimens (houses and animals) were immediately fixed in 2.5% glutaraldehyde-Millipore filtered seawater.

Light and electron microscopy

For replica preparation and electron diffraction analysis, the houses were treated with KOH and $NaClO_2$ solution to remove non-cellulosic materials as described previously (Hirose et al. 1999). A JEM-2000EXII transmission electron microscope was used for observation of the replica and selected-area electron diffraction at an accelerating voltage of 100 kV. Some houses were observed with a polarization and Nomarski differential interference contrast microscope before or after treatment with KOH and $NaClO_2$.

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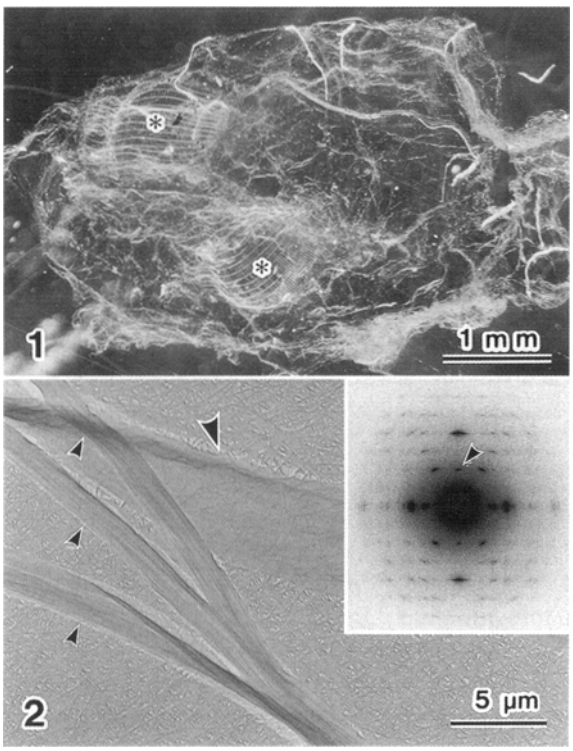


Fig. 1. Dark-field macrophotograph of the house of *O. rufescens* without chemical treatment. The house of *O. rufescens* is a typical oikopleurid house in both size and morphology. The two asterisks indicate the meshwork of inlet filters

Fig. 2. Electron micrographs of the house of *O. rufescens* after treatment with KOH and $NaClO_2$. The insoluble residue from the house is composed of numerous microfibrils. The microfibrils were observed with a random arrangement (background) or as bundles with two different thicknesses (small and large arrowheads). **Inset** Electron diffraction pattern from bundled microfibrils. The diffractograms indicated highly crystalline and an almost pure cellulose Iβ allomorph with a 002 spot (arrowhead)

Results

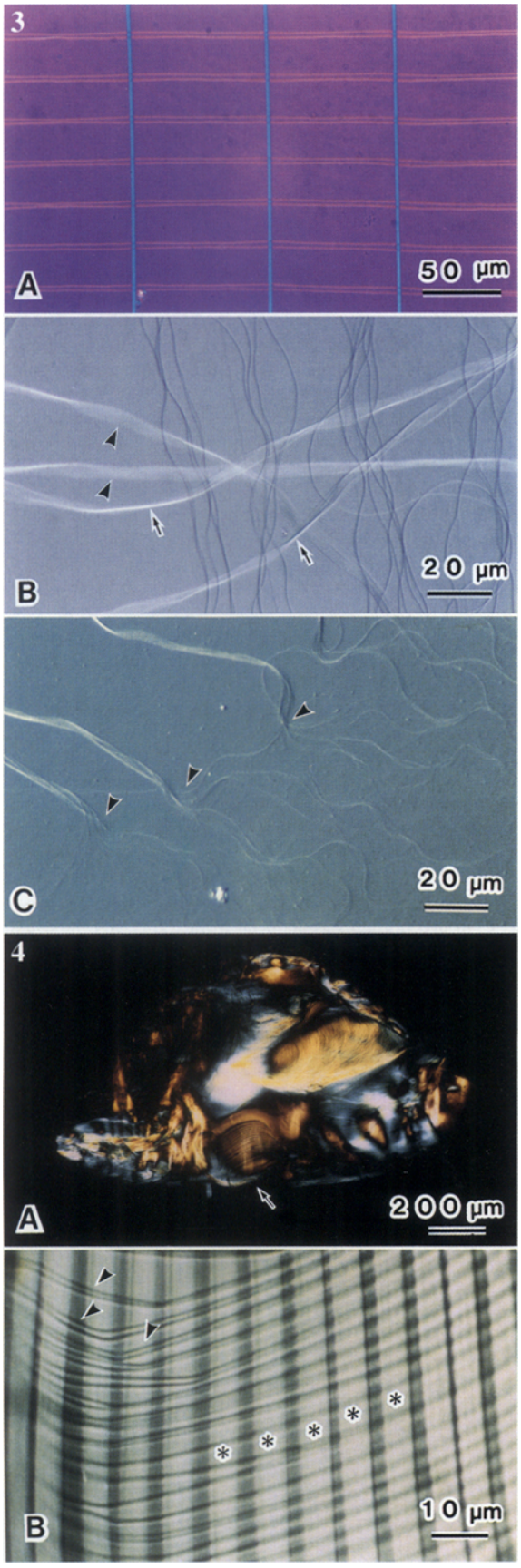
The house of *Oikopleura rufescens* is a typical oikopleurid house, which is a spherical, gelatinous structure and possesses two inlet filters as described by Alldredge (1977) (Fig. 1). The mesh structure of the inlet filters was more stable against mechanical stress than other gelatinous regions of the house before chemical treatment. After treatment with KOH and $NaClO_2$ solutions, insoluble gelatinous materials with an irregular meshwork structure were obtained. The insoluble materials were composed of numerous microfibrils that were randomly arrayed (Fig. 2, background) or bundled (Fig. 2). The former were derived from a gelatinous region and the latter from the inlet filters. Electron diffraction images of the bundled microfibrils showed them to be composed of highly crystalline cellulose I (Fig. 2 inset). The sharpness and strength of the 002 spot derived from a cellulose Iβ allomorph, and the lack of superlattice reflections derived from Iα showed that the cellulose microfibrils were composed of purely crystalline cellulose Iβ (Fig. 2 inset). The diffractograms derived from cellulose Iβ were also obtained from the randomly oriented microfibrils with circular diffraction patterns (data not shown). The mean diameter of individual cellulose microfibrils was 23 nm (n = 50; standard deviation, 3). The width of microfibril bundles was variable from 2 to 10 μm (n = 20). Each bundle with a diameter of 10 μm was calculated to contain more than 430 individual cellulose microfibrils.

The inlet filter of the house formed as a highly ordered meshwork structure similar to a woven textile consisting of a single warp thread 5 μm in width and two tightly closed woof threads 3 μm in width (Fig. 3 A). The two woof threads were woven along the warp at intervals of 22.8 μm (n = 100; standard deviation, 1.7). Thus, the average mesh size of inlet filters was 66.2 by 22.8 μm, comparable with the values reported in Alldredge (1977). The meshworks of inlet filters were unfolded after treatment with KOH and $NaClO_2$ (Fig. 3 B, C). The warp of the inlet filter had a flattened ribbon-shape with a width of 10 μm (Fig. 3 B) and a height of 1–2 μm after treatment. The woof threads maintained the same dimensions after treatment. The terminal structure of the warp was visible after treatment with KOH and $NaClO_2$. The warp was branched into dozens of smaller microfibril bundles at its termini (Fig. 3 C). The branches were entangled with randomly arrayed microfibrils in the house.

The cellulosic component of the house rudiment was obtained from zooids after treatment with KOH and $NaClO_2$. The house rudiment was composed of much more highly condensed cellulose microfibrils than the mature house (Fig. 4 A); the inlet filter is also closely packed (Fig. 4 A). The warp thread with a width of 5 μm was already arranged in order at intervals of 2 μm in the house rudiment (Fig. 4 B). The two woof threads were also already arranged vertically to the warp (Fig. 4 B).

Discussion

Highly crystalline cellulose Iβ was found in the house of the appendicularian *O. rufescens*. Our observations indicated that the cellulose-synthetic ability is a characteristic common to all tunicates, i.e., ascidians,



thaliaceans, and appendicularians. The crystalline features and the dimensions of cellulose microfibril in *O. rufescens* were similar to those of ascidians and thaliaceans (Belton et al. 1989, Daele et al. 1992, Okamoto et al. 1996, Hirose et al. 1999). A similar biosynthetic system such as a linear type of cellulose-synthesizing complex (linear TC), which has been found in the epidermal cells of ascidians (Kimura and Itoh 1996), may be involved in the formation of cellulose microfibrils in the appendicularian house.

The 18S rDNA molecular phylogeny indicated that the tunicates are a monophyletic group and that the appendicularians diverged early from the tunicates (Wada and Satoh 1994, Wada 1998). The present study indicated that the common ancestor of all tunicates already possessed the ability to produce highly crystalline cellulose I. This suggests that other animals close to the tunicates, such as cephalochordates, vertebrates, and hemichordates, might also produce cellulose or possess the cellulose synthase genes. Cellulose synthase genes have been found in bacteria, protists, and land plants (Delmer 1999). The genes have some highly conserved regions and variable regions among species (Delmer 1999). The cloning of cellulose synthase genes of tunicates as well as finding other animals that produce cellulose are required to understand the evolutionary pathway and diversity of cellulose-synthetic ability among the cellulosic organisms. Whereas all ascidians and thaliaceans possess the tunic as an integumentary tissue covering the epidermis, appendicularians have no tunic but have a house as a filter-feeding apparatus. Both the house and the tunic

Fig. 3. Light micrographs of inlet filter in the house of *O. rufescens* before (**A**) and after (**B** and **C**) treatment with KOH and $NaClO_2$. **A** The inlet filter is a highly ordered meshwork structure and composed of cellulose bundles with two ranges of thickness (polarization microscope). **B** and **C** The meshwork of the inlet filter collapsed after chemical treatment (Nomarski differential interference contrast). **B** Thicker cellulose bundles are visible with a flattened ribbon shape (arrowheads) and a twisted shape (arrows). **C** The termini of thicker cellulose bundles branched into smaller bundles (arrowheads) and became entangled with the cellulose of the gelatinous regions in the house

Fig. 4. Light micrographs of the house rudiment of *O. rufescens* after treatment with KOH and $NaClO_2$ (**A**, polarization microscope; **B**, Nomarski differential interference contrast). **A** The house rudiment is composed of cellulose microfibrils with high density, and the small inlet filter was already formed in the house rudiment (arrow). **B** The inlet filter was composed of a closely packed woven textile-like material with a single warp thread 5 μm in width (asterisks) and two tightly packed woof threads (arrowheads)

contain cellulosic components and are secreted by epidermal cells. Therefore, the appendicularian house is suggested to be homologous to the tunic of the other tunicates. While it is uncertain how the cellulose was utilized in their common ancestors, the cellulosic components probably developed as a feeding apparatus in the appendicularian lineage and as a protective integument in the ascidian-thaliacean lineage.

The inlet filter of the house in *O. rufescens* builds up a highly ordered meshwork structure like an elaborately woven textile. The meshwork is extremely precise, with errors in the interval between woof threads of the inlet filter of less than 1.7 μm (7.5% of 22.8 μm). The meshwork is unfolded with chemical treatment, but cellulose microfibrils remain as bundled structures. Some proteins or noncrystalline polysaccharides have been suggested to participate in gluing the cellulose bundles to one another. The oikopleurid appendicularian already has a fully synthesized house rudiment on its trunk, and expands a new house after escaping from the old one (Flood and Deibel 1998). In this study, we showed that the rudiment of the house is composed of condensed cellulose microfibrils. The cellulose bundles in the inlet filter rudiment are of almost the same width, but the bundles of cellulose are more highly packed than the mature inlet filter. It is of interest that the mechanism responsible for constructing such a highly ordered meshwork is a simple process of expansion. The question remains as to how the cellulose bundles in the house rudiment are formed and packed. Further ultrastructural studies of the development of the house rudiment are required to understand the mechanisms of house formation.

Acknowledgments

This work was supported by a Grant-in-Aid for "Research for the Future" Program (no. JSPS-RFTF 96L00605) from the Ministry of Education, Sciences, Sports and Culture of Japan to T.I. We thank Professor R. Malcolm Brown, Jr., of the University of Texas at Austin for critical reading of the manuscript.

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