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Determining the Composition of the Dwelling Tubes of Antarctic Pterobranchs

Senior Honors Thesis
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Abstract. Pterobranchs are a group of marine invertebrates within the Hemichordata, which share characteristics with both chordates and echinoderms. Pterobranchs live in colonies of secreted tubes, coenicia, which are composed of a gelatinous material of unknown composition. Visually, the tubes appear similar to the tunic of tunicates, a group of invertebrates within the Chordata. The nonproteinaceous tunic of tunicates is composed of cellulose, which is unusual. The goal of this study was to determine the composition of the pterobranch coenicium. Some aspects of pterobranch phylogeny are still unclear even after multiple molecular and morphological studies. Identification of any new shared characteristics with either echinoderms or chordates would be valuable in determining clearer relationships among these taxa. Purification methods, histology, and microscopy techniques were used to study the structure and properties of the tube material. To date, the results indicate that that the tube material may be protein but the composition is unknown.

Objectives of this Study

The debate regarding relationships among the deuterostomes (Figure 1) led to a search for characteristics that pterobranchs, as hemichordates, may share with either echinoderms or chordates. New information could further clarify relationships within the Deuterostomia and provide additional information about the ancestor to deuterostomes. This study focused on the composition of the tube material of pterobranchs because this is an area that has not been studied in adequate detail, and currently the composition and structure of pterobranch tubes is poorly understood, even though claims have been made as to the composition (Armstrong et al. 1984, Dilly 1971).

This paper begins with a general background of evolutionary relationships within Deuterostomia, it considers justifications for grouping hemichordates, chordates and echinoderms as deuterostomes, and includes a discussion of how these individual taxa relate to each other to provide an evolutionary background for this research. An overview of Hemichordata explains why two superficially different organisms, the pterobranchs and the enteropneusts, are grouped within one taxon and how this relates to deuterostome evolution and the ancestral deutorostome. Finally, a summary of the current research on pterobranchs and the composition and formation of their dwelling tubes is presented.

Introduction

Evolutionary Relationships Among the Deuterostomia

A long standing debate that has continued for more than a century in the study of animal systematics is the evolutionary relationship among the Deuterostomia, a classification that includes echinoderms (e.g. sea stars), chordates (e.g. vertebrates), and the lesser known hemichordates (Halanych 1995) (Figure 1). The Hemichordata is divided into two classes: the

Enteropneusta and the Pterobranchia (Cannon et al. 2009, Cameron 2005). Enteropneusts, commonly known as acorn worms, are marine burrow-dwelling worms characterized by multiple gill slits and a straight gut with a terminal anus (Figure 2a). Pterobranchs are small (1-5mm in length), marine, colonial animals that live in constructed tubes attached to the benthos (Figure 2b). Hemichordates represent an important branch in the evolutionary tree of deuterostomes because they share multiple characteristics with chordates and echinoderms, and their relationship to these two groups remains equivocal (Ruppert 2005, Halanych 1995, Turbeville et al. 1994). Current research on the relationships between hemichordates, chordates and echinoderms seeks to identify characteristics that are ancestral for deuterostomes (Ruppert 2005).

Establishment of the Hemichordata as a Clade

Superficially, the burrow-dwelling acorn worms do not appear to be related to the colonial tube-dwelling pterobranchs. However, morphological characteristics unite enteropneusts and pterobranchs as part of the Hemichordata. These characteristics include the stomochord, collar region, glomerulus and associated kidney structures, and three paired coelomic body cavities (Halanych 1995, Cannon et al. 2009, Sato et al. 2008, Figure 3a,b).

Based on visual similarity of their tentacular feeding structures, pterobranchs were originally grouped with lophophorates (bryozoans), not the enteropneusts (Cameron 2005, Cannon et al. 2009). Further, both lophophorates and pterobranchs are colonial and live in constructed tubes. Recent morphological and molecular studies of bryozoan and pterobranch feeding structures have shown that these tentacles are not homologous and arose at least twice among Metazoa (Cannon et al. 2009, Sato et al. 2008a, Halanych 1996).

The hemichordate clade is supported by analysis of DNA encoding the 18S ribosomal subunit, the structure of which is conserved among closely related species. This indicates that

the enteropneusts and pterobranchs shared a common ancestor (Halanych 1995). Although the inclusion of pterobranchs and enteropneusts in Hemichordata has been firmly established, relationships among members of this group are still being elucidated (Figure 1).

Relationships Among Members of Hemichordata

Elucidating relationships within Hemichordata has been problematic as a result of conflicting evidence. Some studies point to pterobranchs as basal hemichordates, while others support placing enteropneusts as the basal group. Data from 18S ribosomal DNA analysis suggest that the enteropneusts are paraphyletic and that pterobranchs are a sister taxon to the Harrimaniidae, a group within Enteropneusta. This indicates that enteropneusts are basal, while pterobranchs are a derived clade within Hemichordata. Therefore, based on 18S rDNA analysis, the ancestor to pterobranchs may have been similar to modern enteropneusts (Halanych 1995, Cannon et al. 2009, Sato et al. 2008ab, Swalla and Smith 2008, Winchell et al. 2002, Peterson and Eernisse 2001, Figure 1).

In contrast, 28S rDNA and a morphological study using characteristics shared among hemichordates support pterobranchs as basal hemichordates, rather than a derived clade within Enteropneusta (Figure 1). However, the 28S rDNA data sets are smaller than 18S rDNA data sets and therefore less supported (Cannon et al. 2009, Sato et al. 2008ab, Cameron 2005, Winchell et al. 2002).

Increasing the number of taxa and genes included in analyses utilizing any kind of ribosomal data is important for reducing error. Turbeville et al. (1994) caution against over interpreting ribosomal data because these data often conflict with well supported morphological data. Further investigation is necessary to provide a clearer idea of the relationships within

Hemichordata and to determine whether pterobranchs or enteropneusts more closely resemble the ancestral hemichordate.

Evolutionary Relationships Between Hemichordata and Chordata

The hemichordates were once believed to be protochordates, ancestral to the Chordata (Sato et al. 2008, Ruppert 2005, Cameron 2005). However, the hemichordate and chordate grouping was mainly based on the adult morphology of only the enteropneust clade and is not currently supported by molecular analysis (Ruppert 2005, Winchell et al. 2002). Proposed homologies between hemichordates and chordates include pharyngeal gill slits, a dorsal hollow nerve cord, an endostyle (an iodotyrosine-secreting structure that is the precursor to the chordate thyroid gland), and a stomochord that is similar to the notochord of chordates (Sato et al. 2008, Ruppert 2005, Cameron 2005) (Figure 3c). Bateson (1886) was an early proponent of including Hemichordata in Chordata, but few taxonomists have accepted this hypothesis (Ruppert 2005, Tagawa 2001).

Recent studies have demonstrated that most of the structures originally proposed as homologies between chordates and hemichordates are not actually homologous and that these two groups are not sister taxa. According to Halanych (1995), the presence of a stomochord in pterobranchs has not been adequately demonstrated by microscopy. Ruppert (2005) proposed that the stomochord and notochord are most likely homoplasies, and besides sharing a basic structural role, these structures are not homologous. Most researchers agree that the notochord of chordates and stomochord of hemichordates are not homologous (Sato et al. 2008, Gerhart et al. 2005). Additionally, the dorsal nervous tissue of hemichordates is likely not homologous to the dorsal nerve cord of chordates (Sato et al. 2008, Gerhart et al. 2005). No studies have shown a defined region in hemichordates that could be called an endostyle and more work needs to be

done to determine whether hemichordates have a structure corresponding to the chordate endostyle (Sato et al. 2008).

According to Ruppert (2005), gill slits superficially appear to be homologous, but subtle differences between them suggest that they are not homologous at the secondary level. Most likely the simple primary gill slits are homologous, but complex secondary gill slits are homoplasies (Ruppert 2005). The homology of primary gill slits is supported by the expression of a number of patterning genes, including Pax 1 and Pax 9 (Cannon et al. 2009, Gerhart et al. 2005, Ruppert 2005, Tagawa et al. 2001). Although hemichordates and chordates share gill slits as a homology, most analyses indicate that these two groups are not sister taxa and gill slits must have been an ancestral deuterostome characteristic that was present before hemichordates and chordates diverged. The data presented in this review suggest that the deuterostome ancestor was most likely worm-like, had simple gill slits and was a filter feeder (Sato et al. 2008, Swalla and Smith 2008, Turbeville er al. 1994).

Evolutionary Relationships Between Hemichordata and Echinodermata (Ambulacraria)

The placement of hemichordates and echinoderms as sister taxa within a relatively new taxon called Ambulacraria (Figure 1) is nearly universally supported by molecular and developmental data (Cannon et al. 2009, Sato et al. 2008ab, Swalla and Smith 2008, Ruppert 2005, Winchell et al. 2002, Tagawa et al. 2001, Turbeville et al. 1994, Ruppert and Balser 1986). The discovery of the tornaria, a dipleurula type larva (Figure 4) of enteropneusts, was one of the first events that led to the suggestion that hemichordates and echinoderms are more closely related than previously thought (Sato et al. 2008). Since the discovery of the tornaria, a dipleurula type larva has been considered a synapomorphy for Ambulacraria (Swalla and Smith 2008, Byrne et al. 2007).

A number of embryological characteristics are shared between hemichordates and echinoderms, including larval development, trimeric arrangement and development of coelomic cavities, circumoral larval ciliary bands and an asymmetrically positioned heart-kidney (Cannon et al. 2009, Swalla and Smith 2008, Ruppert 2005, Halanych 1995, Ruppert and Balser 1986).

One of the difficulties in grouping echinoderms and hemichordates is the extensive modification of adult structure and development in echinoderms, which makes molecular analysis important (Swalla and Smith 2008, Tagawa et al. 2001). Data from 18S rDNA and other molecular analysis consistently supports the grouping of hemichordates and echinoderms as sister taxa within Ambulacraria, as do more comprehensive analyses using multiple genes (Sato et al. 2008, Swalla and Smith 2008, Peterson and Eernisse 2001, Tagawa et al. 2001, Halanych 1995). Combining 18S and 28S rDNA data increases the bootstrap values (i.e., values used to determine the probability that a certain taxonomic grouping is likely) supporting the grouping of echinoderms and hemichordates in Ambulacraria to nearly 100% (Winchell et al. 2002).

The existence of Ambulacraria as a taxon is supported by extensive evidence and is currently the most widely accepted grouping for the Hemichordata. The placement of hemichordates within Ambulacraria suggests that some characters shared by hemichordates and chordates are primitive deuterostome characters. These characters must have arisen before Chordata and Ambulacraria diverged and include ciliated gill slits, which have since been lost in echinoderms (Cannon et al. 2009, Sato et al. 2008ab, Halanych 1995). Identifying new characteristics in deuterostomes is important for further developing taxonomic relationships between hemichordates, chordates and echinoderms, and identifying what characters were present in the ancestral deuterostome.

Overview of Pterobranchia (Hemichordata)

Pterobranchs are divided into two genera, *Rhabdopleura* and *Cephalodiscus,* and are believed to be closely related to the extinct Graptolites, which are often grouped as pterobranchs (Sato et al. 2008). Pterobranchs have mostly been collected from deep (50-650m) Antarctic waters, except for some species found in shallow (20-300m) tropical waters (Cameron 2005, Lester 1985). They are rarely collected due to their small size, although more species may be present in tropical areas but have not been found due to their inconspicuous appearance (Lester 1988b 1985, Balser personal observations). Pterobranchs typically live in colonies with multiple coenecia (non-living, hollow, colonial tubes, Figure 5) with one zooid (living member of the colony, Figure 6) in each tube (Sato et al. 2008a, Lester 1985, Cannon et al. 2009, Figure 6). The coenecium is a network of tubular chambers with branching tubes that open to the outside (Lester 1988b, 1985, Mierzejewski and Kulicki 2001). Unlike zooids of *Cephalodiscus* spp, which are connected to a basal disk, zooids of *Rhabdopleura* spp. are connected via a stalk and strands of tissue traversing the colony (Lester 1985, Figure 7). Since deep sea specimens often lack zooids upon retrieval or are poorly preserved, most descriptions of pterobranchs focus on the structure of the coenecium (Lester 1985). This highlights the importance of understanding the composition of the coenecium to developing evolutionary models that involve pterobranchs.

The Coenecium of Pterobranchs: Composition

The coenecium of *Cephalodiscus* spp. and *Rhabdopleura* spp. is translucent or orange due to the color of the zooid and may be embedded with detritus, such as sand or algae, which gives it a brown color (Lester 1988b, 1985, Mierzejewski and Kulicki 2003, Figure 5). The zooid is divided into three regions; the protosome which includes the flat, glandular cephalic

shield; the mesosome, which is the collar region and includes the foregut, oral lamellae and the tentaculate arms, and the metasome, which is the trunk and contains the digestive tract, gonads and stalk (Figure 6) (Lester 1988b, 1985). The ventral epidermis of the cephalic shield is composed in part of tube material secreting cells and is responsible for forming the coenecium (Lester 1988b, 1985).

The chemical composition of the coenecium has been disputed multiple times since pterobranchs were first placed in the class Pterobranchia by Lankester (1877). Kraft (1926) used staining techniques and concluded that the tube material was chitin, although this was disputed by many researchers (Armstrong et al. 1984). Dilly (1971) argued that based on their dimensions, the fibrils in the tubes were composed of a mass of keratin loose in a matrix that has little structure. However, this view was challenged, since the coenecium does not contain the sulfur containing amino acid cysteine, which is necessary for the disulfide bonds that give keratin its exceptional strength (Armstrong et al. 1984).

Analysis of the amino acid content of the coenecium of *Rhabdopleura* and *Cephalodiscus* by Armstrong et al. (1984) led to the generally accepted conclusion that the fibrils were composed of collagen. The amino acid levels are characteristic of invertebrate collagen, including residues (e.g. glycine, proline and hydroxyproline) that are fingerprint markers for collagen (Armstrong et al. 1984). However, the ultrastructure of the fibrils is not consistent with that of previously studied collagen fibers. In specimens of *Rhabdopleura normani*, Dilly (1971) noted that the fibrils were unusual, with small diameters (25-30 μm) and a double helix not found in collagen fibrils (these are normally found as triple helices). Regular banding is a characteristic structure of collagen that is also not present in specimens of *Rhabdopleura normani* or *Cephalodiscus hodgsoni* (Armstrong et al. 1984). The methods used in the study by Armstrong

et al. (1984) do not provide a means to determine whether the collagen is in the ground substance or in the fibrils themselves. Digesting the coenecium and amino acid analysis of the resulting solution does not indicate where the amino acids originate; they could be part of the gelatinous matrix or the fibrils embedded in it. Thus, the chemical composition of the fibrils is unknown.

The Coenecium of Pterobranchs: Structure and Arrangement

Pterobranchs have fusellar and cortical tissue in their coenecium (Swalla and Smith 2008, Armstrong et al. 1984, Mierzejewski and Kulicki 2003, Figure 8). Fusellar tissue in pterobranchs (*Rhabdopleura* and *Cephalodiscus*) contains narrow, straight, unbranched fibrils embedded in a matrix of unknown composition. The fibrils have a lucent central core and a diameter of 40-300 nm (Mierzejewski 1984, Mierzejewski and Kulicki 2003, Figure 8b). In general the fusellar tissue of *Rhabdopleura* and *Cephalodiscus* is very similar (Mierzejewski and Kulicki 2001). The three dimensional network of fibers contains three types of fusellar fibrils that vary in thickness (40-300nm) and length (several nm to 5 μm); the thickest fibrils are arranged as double helices (Mierzejewski and Kulicki 2001).

Dense arrangements of parallel cortical fibrils are found in fusellar collars and also in secondary deposits on the inside of the coenecium (Figure 8). Cortical fibrils are thicker (150-520 nm) than fusellar fibrils (40-300 nm) (Mierzejewski and Kulicki 2003). Secondary deposits are composed of straight, parallel and loosely distributed cortical fibrils and are reported to only be secreted as part of the inner wall of the coenecium (Mierzejewski and Kulicki 2003). Based on transmission electron microscopy (TEM), Dilly (1976) reported that secondary deposits were present on the outside of the coenecium. However, Mierzejewski and Kulicki (2003) demonstrated via scanning electron microscopy (SEM) that these are just wrinkles caused by

shrinkage from drying. The secondary deposits consist of membranous and fibrillar cortical fibrils (Mierzejewski and Kulicki 2003).

Reproduction in Pterobranchs and Secretion of the Coenecium

Asexual reproduction and tube formation in pterobranchs can occur in several ways. Buds (Figure 6) can develop at the base of zooids in *Rhabdopleura* and from the base of the basal disc in *Cephalodiscus*, followed by the formation of a septum to separate the tubes (Sato et al. 2008a, Lester 1985). A zooid can also detach and leave the colony, secrete a new tube and develop into a colony in a different location. If the coenecium is removed from a cluster of zooids of *Cephalodiscus* spp. the zooids will secrete tubes within 24 hours and resume feeding after two days (Lester 1985). Various developmental stages in *R. compacta* can be found within the coenecium and colony morphology and zooid state show seasonal variation (Sato et al. 2008a, Rigby 1994, Lester 1988b).

During sexual reproduction in *Rhabdopleura normani*, embryos incubate up to seven days in the brood chamber and develop into swimming lecithotrophic larvae (Lester 1988ab). In both *Rhabdopleura* and *Cephalodiscus* the free larvae swim for several minutes to 24 hours prior to settling on the benthos (Lester 1988 a,b). During the process of settling, larvae stop as they encounter solid objects and creep over the surface to determine the suitability of the substrate. After selecting a site in which they will settle, each larva begins to revolve and encapsulates itself in a colorless, ellipsoidal cocoon (Lester 1988ab, Figure 9). The cocoon is most likely secreted by secretory cells in the ventral epidermis, which will eventually become the ventral surface of the oral shield (Rigby 1994, Lester 1988a). After the first hour, the cocoon begins to resemble the adult coenecium (Lester 1988a). The larva may remain in the cocoon for several weeks to several months before metamorphosing to an adult zooid (Sato et al. 2008, Lester

1988a, Balser personal observations). To create the opening to the coenecium, the larva secretes a substance that removes material from the top of its cocoon (Lester 1988a). Both the cocoon and the adult coenecium are flexible and translucent brown, which suggests that they may have a similar composition (Lester 1988a).

Coenecium of Pterobranchs: Personal Observations

The dwelling tube of the pterobranch *Cephalodiscus nigrescens* is made of a clear, gelatinous substance (personal observations, Figure 10a). The material is durable and does not degrade easily, even when it is left at room temperature for several days, and can be kept in a refrigerator for multiple years without visible signs of degradation (observations by E.J. Balser). This tube is in many ways similar to the ascidian chordate tunic (Figure 10b), which is composed of tunicin, an animal form of cellulose (Nakashima et al. 2008). Ascidians, commonly called tunicates, are a group of filter feeders within chordata and the presence of tunicin is a characteristic feature for this group. Both the dwelling tubes and the tunic are durable and resistant to degradation and their appearance is similar. Cellulose was initially considered as a potential structural component for the pterobranch dwelling tube based on these similarities.

Purpose

The purpose of this study was to determine the composition of the dwelling tubes of the Antarctic pterobranch *Cephalodiscus nigrescens*. The composition of the dwelling tubes is unknown and although previous studies have attempted to identify the fibrils in the coenecium, no strong evidence has been proposed in favor of one material. Due to similarities between the dwelling tube and the tunic of *Ciona intestinalis*, an ascidian chordate, cellulose was considered as a possible structural material. Chitin, keratin and collagen were also considered on the basis of previous research and knowledge of common invertebrate structural components. Identifying

the structural component of the coenecium would be useful for comparing how the tube material of pterobranchs relates to the structural components of other deuterostomes, which could provide additional data to clarify relationships among members of this taxon. Staining, purification and IR techniques were used to investigate the structural composition of the pterobranch coenecium.

Methods

Collection of Samples

Pterobranch colonies were collected by E.J. Balser from waters surrounding the north western quadrant of Antarctica in the vicinity of the Shetland Islands in May of 2006. Specimens were dredged from various depths (75-200 meters) using a Blake trawl operating from the Lawrence M. Gould research vessel. Some specimens were frozen at -80 ^{o}C, others were fixed for microscopy. Preparation of pterobranchs varied for each protocol and is outlined in the following sections.

Calcofluor White Staining of Frozen Sections

According to Bhavasar et al. (2010), Calcofluor White (CW) (Fluka Analytical 18909) can be used as a differential stain for β1-3 and β1-4 linked polysaccharides. Beta 1-4 linked polysaccharides include cellulose, which is found in plant cell walls and in tunicates, and chitin, which is found in the exoskeletons of many invertebrates as well as the cell walls of some bacteria and fungi. Calcofluor White is combined with Evans blue as a background stain. Cellulose fibers stained with CW fluoresce with long-wave UV light and short-wave visible light (Bhavasar et al. 2010). Stained sections of pterobranchs were evaluated with a Nikon Eclipse E600 microscope and photographed with a Nikon Digital Sight M306E camera.

This staining method was tested on several organisms to determine whether the stain would differentiate types of fibers in organisms other than fungi. Specimens were used with

fibers for which the composition is known: annelids (collagen), chicken leg (mixed: keratin, collagen), marine algae (cellulose), pterobranchs (unknown) and tunicates (tunicin). The tunic was removed from a tunicate (*Ciona intestinalis*) and sectioned with a razor blade. Annelids were euthanized in the freezer and cross sections were taken with a razor blade. Sections of an unknown alga from the seawater tanks in the Center for Natural Sciences at Illinois Wesleyan University and sections of tissue from a chicken leg, acquired from a local grocery store, were obtained by hand sectioning material with a razor blade. Cryostat sections of pterobranchs were also used later in the study.

Sections from all of the above sources were placed on microscope slides; one drop of 5% aqueous KOH and one drop of Calcofluor White were added directly to the samples which were placed in the dark for one minute. After one minute the samples were rinsed with Nano Pure water and placed in the dark until examination using the fluorescence microscope.

Extraction of Cellulose and Chitin for Infrared (IR) Spectroscopy Analysis

A protocol for the purification of tunicin (cellulose) in ascidian tunicates by Nakashima et al. (2008) was adapted to examine pterobranch tubes for the presence of cellulose. Initially, tunicates were used to test the reliability of the protocol.

The tunic was removed from living specimens of *Ciona intestinalis*; non-tunic debris was removed and the sample was rinsed with Nano Pure water. All samples were dried in an oven at 60 °C or freeze dried overnight in a Granville-Phillips Ultra Dry freeze dryer connected to an Ulvac GVD 050A vacuum pump. After drying, samples were placed in a 5% aqueous KOH solution at 37 ^{o}C overnight. Upon removal from the KOH solution, specimens were placed into 1% aqueous acetic acid for 6 hours to neutralize the KOH. The samples were then washed with water and transferred to a 0.35% aqueous solution of NaOCl in an 80 ^{o}C water bath for two

hours in order to bleach the specimen; this bleaching procedure was repeated up to three times or until the sample was clear/white. Once the samples were sufficiently bleached or appeared to have a whitish color they were removed from the NaOCl solution and placed into a mixture of 9% aqueous HNO_3 and 73% aqueous CH_3COOH in a water bath at 95 ^oC for thirty minutes in order to break down any remaining non-cellulose molecules. After every step the samples were thoroughly rinsed using Nano Pure water. The samples that remained after the procedure were frozen in a -80 ^oC freezer and then freeze dried.

The freeze dried samples were analyzed using the attenuated total reflection (ATR) IR spectroscopy technique, which is used for solid state samples, in a Thermo Scientific Nicolet iS10 SMART iTR IR Spectrometer. Cellulose samples were compared to a powdered cellulose standard.

To determine the specificity of the test for cellulose and not chitin, the cellulose extraction protocol was applied to extract chitin from bryozoans, crab and crayfish legs, and the egg sac of the Chinese praying mantis, *Tenodera aridifolia sinensis*. This was to ensure that the protocol is specific for cellulose and not chitin, which is a common invertebrate exoskeleton component and contains the same β1-4 linkages as cellulose.

For crabs and crayfish, legs were removed from live animals and rinsed with Nano Pure water to remove non-exoskeleton tissue. The praying mantis egg case was cut in half to fit it into the test tube. The samples were air dried or freeze dried and chitin was extracted using the same protocol as the one used for cellulose. Following extraction, samples were freeze dried. The exoskeleton samples were analyzed using IR spectroscopy as above and the resulting spectra were compared to a literature spectrum. For bryozoan samples, the importance of the final

nitric/acetic acid step was tested by removing one set of samples after bleaching with NaOCl and freeze drying them immediately. The other samples were also subjected to the entire protocol.

For pterobranchs, a section of the colony was removed using scissors and visible algae and non-pterobranch organisms were removed from the tubes. The samples were then rinsed with Nano Pure water and either air dried or freeze dried. The dry samples were then purified using the cellulose extraction protocol of Nakashima et al. (2008) and freeze dried for IR spectroscopy. Some pterobranch samples were put through the entire protocol and some were removed and freeze dried following the NaOCl bleaching step. For later trials, the last step of the pterobranch extraction procedure was carried out in a microfuge tube in order to preserve as much of the remaining sample as possible.

Trichrome Staining of Frozen and Epon Embedded Sections of Pterobranchs

Trichrome stains have been used to differentiate various macromolecular components of a sample, such as proteins (McManus and Mowry 1961, Gray 1954). Hollande's and Masson's Trichrome stains were chosen because of their ability to differentiate collagen and keratin, which are proteins that have been identified as potential structural components of the pterobranch coenecium (Armstrong et al. 1984, Dilly 1971). The Masson's procedure described by McManus and Mowry (1961) was used in this study. The Hollande's procedure was taken from Gray (1954) and originally described by Hollande (1912).

Hand sectioned material was used to initially determine whether the samples would stain, but they proved to be too thick. Either material embedded in epon (a type of plastic used for sectioning) and cut using an ultramicrotome, or frozen material cut using a cryostat was used. Initial cryostat sections crumbled and did not adhere to the slide. Frozen samples were impregnated with Uncle Ben's Secret Sucrose Solution to provide cleaner cryostat sections. The

solution was prepared by adding monobasic sodium phosphate (1.38 g) and dibasic sodium phosphate (7.7 g) to 500 mL of water. The solution was placed on low heat and sucrose (150 g) was added. The mixture was vigorously stirred until all solute dissolved and the resulting solution was stirred continuously while on a hot plate for an additional 15min. Samples were impregnated with the sucrose solution by first fixing them in a solution of paraformaldehyde overnight and then placing them in the sucrose solution until samples sank.

Staining was performed directly on slides. Zein (a corn starch derivative) was initially used to attach the samples to slides, but it is alcohol soluble and did not remain adhesive throughout the procedure. As an alternative, a gelatin slide preparation technique described by Kiernan (1999) was used. The gelatin solution was prepared using K_2CrO_4 (0.175 g), 250 mL H_20 and gelatin (2.5 g). The solution was heated on a hot plate and the slides were immersed in the solution once the gelatin was completely dissolved. The slides were left in the gelatin for 5 minutes and then set out to air dry for several hours. Sectioned samples were placed on the coated slides and allowed to dry completely.

Samples for the Masson's staining were placed in 5% aqueous ferric alum for 30 minutes at 50 ^{o}C. The solution was removed and samples were stained in Regaud's hematoxylin (1 g hematoxylin, 10 mL 95% aq. ethanol, 10 g glycerin, 80 mL water) for 30 minutes at 50 ^{o}C and then washed with Nano Pure water. Picric acid in 95% ethanol was used to differentiate the stain and then samples were rinsed with water for several minutes. Acetic acid fuchsin (1 g acid fuchsin, 1 mL acetic acid, 200 mL water) was added to the samples and after sitting for 5 minutes, they were rinsed with water. Aqueous phosphomolybdic acid (1%) was added to the sample for 5 minutes, then, without washing, a saturated solution of aniline blue in 2.5% acetic

acid was added to stain collagen. Samples were rinsed and aqueous acetic acid (1%) was added for 15 minutes to remove excess aniline blue.

For Hollande's staining, samples were placed in a solution of 1% basic fuchsin in 70% ethanol for 6-12 hours (usually overnight). Samples were rinsed with Nano Pure water for 5 minutes. A 0.1% solution of HCL in 70% ethanol was used to decolorize the sample for several seconds (until clouds of dye stopped diffusing into the solution). Samples were rinsed with water and immersed in a 1% aqueous solution of phosphomolybdic acid for 5 minutes and then rinsed with water. A saturated solution of aqueous orange G (CAS # 1936-15-8) was added to the samples for 5 minutes and removed. Without rinsing, a 0.2% aqueous solution of light green SF yellowish (CAS # 5141-20-8) was added to the sample and allowed to sit for 60 seconds. Samples were then rinsed with water and examined using a compound microscope.

Results

Calcofluor White Staining of Frozen Samples

Analysis of known samples of various structural fibers indicates that Calcofluor White was not specific enough to differentiate cellulose fibers from other materials in the samples. According to the original procedure, fluorescent fibers on a blue background should have been visible in cellulose and chitin containing structures. Since all samples showed staining of fibers, this indicated that the stain was not only staining cellulose, but also collagen and potentially other protein fibers (Figure 11). Calcofluor white staining of pterobranch tubes did not make it possible to visualize the fine structure of fibrils in the coenecium (Figure 12). Only the frozen specimens were used in this procedure, since epon plastic blocks UV light, and therefore samples would not fluoresce. The gelatinous nature of the coenecium made hand sectioning very difficult and thin, evenly cut sections could not always be obtained reliably.

However, some sections through pterobranchs did show the structure of the tubular network (Figure 11a). Cryostat sections yielded mixed results. Although tubes and fibers could be visualized in some cases (Figure 12), many of the sections fell apart on the slides or as they were transferred to the slides. Sucrose embedded sections were originally not considered suitable for staining with CW due to the presence of β1-4 linkages in sucrose, which would interfere with the staining in sections. However, CW staining of sucrose embedded sections did not show additional background staining (Figure 12); this could potentially be a result of the water solubility of sucrose, which allowed it to wash away along with the stain.

Extraction of Cellulose for IR Analysis

Cellulose was extracted from the tunicate *Ciona intestinalis* and confirmed by matching the spectrum with the IR spectrum of a sample of pure powdered cellulose (Figure 13). The sample was extracted as one piece, demonstrating the extent of the cellulose network and was difficult to powder, but could be separated into sheets.

Chitin was also successfully extracted from bryozoans, crab and crayfish legs and an egg case of the Chinese praying mantis. The presence of chitin was confirmed using IR spectroscopy and compared to a reference spectrum (Figure 14). The final step of the procedure is essential for obtaining a clean IR spectrum. Removing the bryozoan samples after the NaOCl bleaching step resulted in additional peaks in the spectrum that were not present in the spectrum for samples that were treated with the nitric/acetic acid solution.

The extraction of a pterobranch tube resulted in disappearance of most of the sample during the final step of the procedure. The sample gradually dissolved as it was treated with nitric acid and acetic acid. Upon examination using a microscope, the sample that remained after this step was white and fibrous in appearance, but did not form a network. IR analysis of the

remaining sample was inconclusive and the tube material could not be confirmed as cellulose or chitin (Figure 15).

Trichrome Staining of Frozen and Epon Embedded Sections of Pterobranchs

The material in epon fixed sections (1 μm) did not stain effectively; not all of the stain penetrated the samples, which did not allow any conclusions about the nature of the fibers to be made (Figure 16). However, the animals in the tubes stained brown. The material in the cryostat sections (50 μm) took up stain (Figure 17,18) and at high power (100X) fibers appeared to be visible. However, none of these fibers appeared to stain, but were embedded in tissue that generally stained (Figure 17c, 19a,b). Although the background staining is inconsistent for both Masson's and Hollande's stains, every time fibers could be visualized, they were unstained. Additionally, the slides usually stained as well, often obscuring the samples.

Hand cut sections stained with Masson's and Hollande's trichrome stains showed staining, but the unevenness and thickness of the sections prevented the stains from completely and evenly penetrating the samples. This did not allow for any specific conclusions to be drawn regarding the identity of the fibers. The staining times listed in the protocols used in this study were designed for thin sections; longer times may be necessary for thicker samples such as the ones used here. Various techniques were used to stain hand cut sections. Some samples were stained directly on slides, but this was not effective as the samples washed away without any adhesive. Some samples were stained in petri dishes but this was difficult because samples were more spread out and during washing some were washed away. The most effective technique was to immerse all of the sections in small beakers of solution and then transfer them as needed using forceps. This is only practical for hand cut sections due to their large size, which makes them

easy to manipulate; thinner cryostat and microtome sections are prone to tearing and cannot be manipulated extensively.

Originally, frozen samples that had not been impregnated with sucrose were used for cryostat sectioning. This technique did not work because the frozen sections crumbled as they were cut and no clear tissue structure was visible. Sucrose impregnated samples provided cleaner sections but tissue structure was still unclear and few of the sections showed intact dwelling tubes or zooids. This indicates that a better sectioning technique may be necessary.

The corn starch derivative Zein is an adhesive used to attach sections to slides for staining, but for the purposes of this study, it did not work because it is alcohol soluble. In original trials using Zein, samples stained with Masson's trichrome were the most likely to remained attached to the slide, although several samples per slide would always detach. Gelatin is very effective at sticking samples to slides and it is resistant to all of the treatments that were used. However, gelatin may not be a suitable adhesive for stains that detect proteins because gelatin is proteinaceous and picks up stain, which was evident in this study (Figure 17,18). The background stain interfered with the interpretation of results by obscuring any color in the samples.

Discussion

The composition of the coenecium of pterobranchs has been the subject of much debate. Several materials (chitin, keratin and collagen) have been proposed as the major constituent. The methods used in this study tested for all three of these structural components, as well as one that has not previously been considered, cellulose. This study attempted to identify the structural material in the dwelling tubes of pterobranchs using Calcofluor White, Hollande's and Masson's trichrome staining, purification, and IR spectroscopy techniques. The current results did not

indicate any specific material but made it possible to rule out several possibilities, which are outlined below.

The Calcofluor White (CW) procedure did not work as had been anticipated. The staining of multiple samples (chicken legs, algae, arthropods, bryozoan, tunicates and pterobranchs), only some of which are known to contain β-linked polysaccharides, could indicate that the CW stain is not specific for cellulose as reported in the literature. The protocol adapted for use in this study describes CW as a preferential stain for β-linked carbohydrates and utilized CW staining to visualize bacteria and fungi (which contain cellulose) in vertebrate tissues (which do not contain cellulose), but not for the purpose of identifying specific types of fibers (Bhavasar et al. 2010). It is likely that the protocol did not work in this study because CW was used in an attempt to identify a specific type of fiber. The generalized staining observed in this study could have been due to the stain binding to proteoglycans within the matrix of structural fibers. If this is the case, the staining of fibers in a collagen-containing cuticle may indicate that the stain binds to carbohydrate side groups of proteins. These results indicate that CW is an effective stain only if used for the purpose of visualizing and characterizing fibers or for the identification of chitin and cellulose containing organisms as contaminants (Bhavasar et al. 2010). As such, CW could be used to measure and describe the fibers of pterobranchs but not to identify their composition.

The dissolution of the pterobranch sample during the final step of the cellulose extraction procedure could be interpreted in several ways. If the fibers are polysaccharides, then the results of this study indicate that they are not cellulose or chitin. In plants, cellulose is embedded in a matrix of polysaccharides that include hemicelluloses and pectins. These polysaccharides have not been reported in animals. Most reports of structural carbohydrates in animals are of cellulose and chitin, although these fibers are embedded in a matrix of other materials such as protein

(Bhavasar 2010, Nakashima et al. 2008). The data presented here suggest that further studies investigating samples that are known to contain keratin and collagen should be done in order to observe the behavior of these fibers during the extraction procedure. Additionally, the fibrous nature of the remnants left after the procedure suggests that additional tests could be performed on this extract, such as testing solubility in organic solvents or testing for presence of carbohydrates. If this material is fibrous, this would indicate that these are not just debris and could be fibers that originated in the animal.

Masson's and Hollande's trichrome stains are reported to be specific for keratin and collagen (McManus and Mowry 1961, Gray 1952). The diffuse background staining and unstained fibers evident in trichrome stained sections indicate that the gelatinous tissue is likely proteinaceous but do not indicate whether the fibers themselves are composed of protein (Figure 17c, 19). Samples of arthropods (chitin), annelids (collagen) and tunicates (tunicin) could be used to further test the staining properties of Masson's and Hollande's stains. The interference caused by gelatin coated on slides indicates that a different method of adhering sections must be used. Better sectioning techniques are also necessary to obtain sections that show natural tissue structure and arrangement.

Cellulose and chitin are unlikely candidates as the structural material. Trials using multiple cellulose and chitin containing tissues demonstrated that the extraction procedure of Nakashima et al. (2008) is consistent for chitin and cellulose. The effectiveness of this procedure on chitin is not surprising due to the similar linkages within chitin and cellulose, and the durability of chitin as a structural fiber. According to Stankiewicz (1997), chitin and protein are most commonly the structural components of invertebrate cuticles. The fact that chitin is the most common carbohydrate material for structural fibers and cellulose has been found in a

limited group of animals (tunicates) suggests that the tube material may not be a carbohydrate. Additional tests for proteins and carbohydrates, such as the Tollen's test for reducing sugars, would be useful for confirming this.

Collagen and keratin have both been considered as possible components of the fibrils in pterobranchs (Dilly 1971, Armstrong et al. 1984). As a result, these materials were chosen as potential protein structural components of the coenecium. In addition to distinguishing between collagen and keratin, trichrome stains were used to determine whether the structural material of the pterobranch coenecium is a carbohydrate or a protein.

An important question that remains unanswered is why the tube material is so durable. Proteins in exoskeletons are rapidly degraded in most natural environments (Stankiewicz et al. 1997), but the tube material retains its integrity even when left exposed to the air in a non-sterile environment for long periods of time (personal observations, E.J. Balser). If the tube material is collagen, this property would be unusual. Keratins form resistant fibers due to the strong interactions between fibers and numerous disulfide linkages resulting from high cysteine content. Likewise, cellulose and chitin fibrils have a high degree of hydrogen bonding between them, which makes these fibers very durable and resistant to chemical destruction. Additionally, both cellulose, the structural polymer in ascidians, and chitin, the structural polymer in many invertebrates, do not degrade as easily as protein fibers due to the β 1-4 linkages, which cannot be digested by most organisms since they do not possess the proper enzymes. All organisms have proteases, many of which can attack a wide range of proteins. Since few organisms contain cellulase or chitinase, these fibers are not easily degraded, even under non-sterile conditions, and therefore they retain their integrity for longer than many proteins. The results of this project

indicate that more needs to be done to determine the composition of the pterobranch dwelling tube.

If the structural material is a protein, antibodies to common structural proteins could be used and then detected using fluorescence microscopy. The sample could also be subjected to purification protocols for several common structural proteins. Although IR spectroscopy is a good method for confirming the identity of a sample it is less useful for identifying an unknown, potentially novel compound. A better technique would be nuclear magnetic resonance (NMR) spectroscopy but this still requires a pure sample, which cannot be obtained without an appropriate extraction procedure. Stankiewicz (1997) used pyrolysis-gas chromatography in his study on the exoskeletons of extant and extinct invertebrates. This technique could potentially be applied to pterobranchs. While the results of the current study do not make identification of the compound possible, they lay a foundation for future work in this field.

Conclusion

The composition of the dwelling tubes of pterobranchs remains unknown, although this work indicates that this material is most likely not cellulose or chitin. Staining to identify keratin and collagen was difficult to interpret and further work is required to determine whether these proteins are part of the coenecium. Identifying the structural material would provide an explanation for the unusual properties observed in samples of coenecia, especially their resistance to breakdown in the presence of natural decomposers. Future studies could focus on improved staining techniques, fluorescent antibody tagging and pyrolysis/gas chromatography.

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