First report on chitinous holdfast in sponges (Porifera)

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A holdfast is a root- or basal plate-like structure of principal importance that anchors aquatic sessile organisms, including sponges, to hard substrates. There is to date little information about the nature and origin of sponges’ holdfasts in both marine and freshwater environments. This work, to our knowledge, demonstrates for the first time that chitin is an important structural component within holdfasts of the endemic freshwater demosponge *Lubomirskia baicalensis*. Using a variety of techniques (near-edge X-ray absorption fine structure, Raman, electrospray ionization mass spectrometry, Morgan–Elson assay and Calcoflour White staining), we show that chitin from the sponge holdfast is much closer to $\alpha$-chitin than to $\beta$-chitin. Most of the three-dimensional fibrous skeleton of this sponge consists of spicule-containing proteinaceous spongin. Intriguingly, the chitinous holdfast is not spongin-based, and is ontogenetically the oldest part of the sponge body. Sequencing revealed the presence of four previously undescribed genes encoding chitin synthases in the *L. baicalensis* sponge. This discovery of chitin within freshwater sponge holdfasts highlights the novel and specific functions of this biopolymer within these ancient sessile invertebrates.

1. Introduction

Environmental characteristics are known to influence the gross morphology of many benthic organisms including sponges (Porifera; [1,2]). This variability in shape is the consequence of both strategic (during a long-term evolutionary response to environmental pressures) and tactical (in response to local environmental pressures) processes. At local scales, morphology correlates with wave action, current flow rate and sedimentation for both a number of sponge species and entire sponge assemblages [3–6]. Wave action and current flow rate have direct influences not only on sponge morphology, but also on the strength of attachment to substrata.
by its holdfast. For example, wave action is known to affect sponge morphological types, as many of the more delicately branching species are destroyed by drag [4,5]. Therefore, the limits of morphological adaptation for any particular sponge species may reduce species richness at sites of high wave exposure flow. Wave action removes delicate sponge forms such as the pedunculate and arborescent shapes. Encrusting and robust species are more suited to this environment [7]. However, the biomechanical basis for such morphological changes has rarely been documented [5]. The holdfasts of sponges that live in muddy substrates often have complex tangles of root-like growths, however the holdfasts of organisms that live on smooth surfaces (such as the surface of a boulder) have the base of the holdfast literally glued to the surface. For example, the globular demosponge Ciocalyptia subterrana, van Soest & Sass [8] has been found on vertical walls and the floors of caves flooded with water at marine salinity levels [8]. Attachment to the rock is accomplished not by a root of spicules, but by a smooth, flat, disk-like holdfast. Some sponges, e.g. species of Teritentium, may show adaptation to both soft bottom and hard substrate [9].

Detailed analysis of the literature with regard to the nature and origin of the poriferan holdfast suggests that it is a complex structure that is initially developed by the larvae. When a sponge begins its life cycle, it is a microscale free-swimming larva in the water column. The tiny sponge must settle down on a substrate and establish a niche for itself to survive. After settlement, the larvae metamorphoses and begin to transform their organization into the adult body plan. During this process, the outermost layer of cells (the pinacoderm) covers the metamorphosing sponge, as well as the adult body [10]. The basal pinacoderm (basopinacocytes) secretes a mixture of spongin (collagen-like protein) and complex carbohydrates (probably in the form of a fibrillar spongin–polysaccharide complex) that allows the animal to attach to a substrate [11]. The spongion attachment plaques can be seen as the precursor of the sponge holdfast: it is secreted by basopinacocytes, and the protein–carbohydrate-based glue secreted by these cells holds the sponge in place. According to the traditional point of view, spongion is the basic component of the sponges’ organic skeleton. Taxonomically, spongion is a character of the class Demospongiae, which comprises the highest number of known species (currently more than 8000). Only the representatives of marine demosponge Order Verongida possess skeletons that consist mostly (up to 70%) of chitin, and not spongion [12,13].

It was proposed [14] that spongion sticks the animal to its substratum [15], links its skeletal spicules together, and is also present within the coat of sponge gemmules [16]. Although the spongion matrix has been defined as an exoskeleton [16], spongins exhibit different morphological aspects among demosponges and vary according to the tissues. It is currently not known if all spongion assemblies are equivalent [11,17], or whether or not they are entirely made of short-chain sponge collagens [14]. Thus, according to traditional point of view the reticulate skeleton in most demosponges arises from the basal spongion plate [18] and their spicules (if present) are cemented by varied amounts of spongion in the form of bundles and networks.

Formation of the spongion attachment plaques (= anchoring layer, basal layer and spongion lamella) as the possible precursor of the sponge holdfast has been investigated during aggregate differentiation in demosponge cells [15] and for settlement and metamorphosis of the parenchymella larvae [19,20] for freshwater sponges, including the metamorphosed larva stage.

According to observations by transmission electron microscopy and scanning electron microscopy, fibrous material is found within the basal plates of young sponges, and within the holdfasts of adult ones. Intriguingly, to our best knowledge, to date no reports exist containing detailed bioanalytical investigations confirming that the fibrous material observed is really collagen-like spongion. However, from a methodological point of view, verification of the presence of spongion within skeletal formations is very simple. This arises from the excellent solubility of spongion in alkaline solutions. This property of spongion is well known, and was first described by Kunike [21]. Our preliminary investigations attempted to isolate peptides from the spongion-based skeletons of different demosponges. These studies show that the alkali solution hydrolyses spongion. Obtained is a hydrolysate of amino acids with no residual peptides visible on SDS-PAGE gels after staining with Coomassie and the very sensitive silver stain. These results agree well with those reported previously [22] about the strong insolubility of spongion, which aimed to appropriate peptides for proteomics research.

Our recent findings of chitin within skeletons of both marine demosponges from the Order Verongida [12,13] as well as of hexactinellids [23] relied on the fact that spongion, in contrast to chitin, is soluble in a 2.5 M NaOH solution. Therefore, we used this simple test in the present study. We decided to use the endemic freshwater sponge Lubomirskia baicalensis (Pallas, 1773) for these investigations (figure 1a), as the grey or brownish coloured holdfast of this sponge is particularly visible after it has been detached from stones or other rocky substrates (figures 1b and 2a; electronic supplementary material, figure S1). Initial experiments showed dissolution of the holdfast-containing skeletal fragments after 2–4 h in 2.5 M NaOH at 37°C. The presence of residual fibrous matter could also be seen which strongly resembled the shape of the sponge holdfast (figure 2; electronic supplementary material, figures S3 and S4). Isolation of the holdfast in the form of an alkali-resistant fibrous material still containing sileous spicules motivated us to carry out, to our knowledge, the first ever detailed analytical, biochemical and genetic investigations to identify chitin as a possible candidate as the main structural component of the sponge holdfast.

2. Material and methods

(a) Sponge samples

Specimens of L. baicalensis were collected in Lake Baikal near Bolshie Koty Settlement (51°54′12″N, 105°06′00″E) from 15–25 m depths (water temperature 3–4°C) by SCUBA during 2009–2012. The samples collected were placed immediately in containers with Baikal Lake water and ice, and transported to the Limnological Institute SB RAS (Irkutsk) for 1.2 h at a constant water temperature (3–4°C).

(b) Isolation of chitin-based holdfast

The specimens of L. baicalensis were initially carefully inspected for the intactness of their skeletons, and the presence of macroalgae or invertebrates, using a stereomicroscope. Neither contaminants nor damage were observed for the collected species. The isolation of the chitin-based holdfast was performed according to the alkali-based treatment steps as described in the electronic supplementary material in details.
Analytical methods like Raman spectroscopy, near-edge X-ray absorption fine structure (NEXAFS) spectroscopy, Calcofluor White (CFW) staining, as well as electrospray ionization mass spectrometry (ESI-MS) and estimation of N-acetyl-D-glucosamine (NAG) contents are represented in the electronic supplementary material. Specimens of *L. baicalensis* to be used for RNA isolation were frozen in liquid nitrogen; those to be used for DNA isolation were stored in 70 per cent ethanol at 4°C. Total genomic DNA from sponge tissue was extracted using the PureLink Genomic DNA kit (Invitrogen).
Total RNA was isolated from fresh or deep-frozen sponge specimens using a Trizol Reagent kit (Sigma). cDNA was synthesized using a Reverta kit (AmpliSens, Russia). Comparison of the known chitin synthase mRNA sequences of freshwater sponge Spongilla lacustris (HQ688146; HQ686147) and marine sponge Amphimedon queenslandica (XP_003385441) revealed highly conserved regions which have been chosen for designing several degenerate primers (see the electronic supplementary material). PCR products obtained with the primer pair of ChsFW_L1 (5'-GGACATGTTGGATTCTGATTCGAGCAGCAGCTGAACTC-3') and ChsFW_R4 (5'-CTCCGTTGATGACGGCAGCTGAACTC-3') was subsequently cloned and sequenced (see the electronic supplementary material).

Chitin synthase (CHS) genes were identified by comparison with the CHS sequences registered in GenBank using ‘BLAST-X’ tools at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov). The amino acid sequence encoded by the obtained CHS cDNA was deduced using the software EinnSeq (DNAStar Inc, USA). The sequences obtained in this study were submitted to GenBank and can be retrieved under the accession nos JX875071–JX875074.

3. Results
(a) Structural peculiarities of the Lubomirskia baicalensis holdfast

Habitat conditions of Baikal sponges differ considerably from those of other freshwater sponges owing to hydrological and hydrochemical peculiarities of Lake Baikal, such as great depths, long ice periods, low water temperatures in summer (10–12°C) in the upper layers, high oxygen content and low concentrations of organic matter [12,13,23,27]. Lubomirskia baicalensis (figure 1) has a branched shape and an encrusting base with erect (30–60 cm up to 1 m high) dichotomous branches with rounded apices. The diameter of branches varies from 1 to 4 cm ranging from cylindrical to flattened shapes. The colour of live specimens is brilliant green, which is due to the symbionts inhabiting the external layer of sponges [25]. Ectosomal skeleton consists of spicule tufts from primary spongine fibres. The spicule skeleton consists of megaaxes oxeas, uniformly spined (145–233 × 9–18 μm; [26]). Lubomirskia baicalensis is common on rocks, boulders and wood along the entire shoreline at a depth from 3–4 m to more than 50 m. Sponges could be easily mechanically detached from the sponge body (figures 1a; see also electronic supplementary material, figure S1). Initially, we used insertion of selected L. baicalensis specimens into 2.5 M NaOH at 37°C to examine the chemical stability of the sponge skeletons to alkali treatment, because it is a well known fact that spongine can be easily dissolved in solutions that are up to 5 per cent alkali even at room temperature [21]. This stands in contrast to chitin, which is resistant to similar alkali treatment at temperatures of up to 50°C [12,13,23,27].

The light microscopy image (figure 2a) of L. baicalensis holdfast after 12 h incubation in alkaline solution shows the presence of residual siliceous spicules as well as brownish pigmented organic matter with some mineral microparticles (figure 2c) that are resistant to the treatment. This organic material remains undissolved in 2.5 M NaOH, even after incubation over 7 days at 37°C, and shows very intense characteristic fluorescence of chitin after specific CFW staining (see the electronic supplemental material, figure S2). Siliceous spicules, however, are not more visible after this treatment in contrast to some mineral particles. We observed that the alkali-resistant mineral microparticles of the substrate origin are still tightly bound into chitinous fibres (figure 2c; electronic supplementary material, figure S2). Because of this strong incrustation of the chitinous fibrillar network with the mineral phase, we assume that chitin and not spongine, which was dissolved during insertion of the holdfast into alkaline solution, may be responsible for attachment of the sponge to the rocky substrate.

The treatment of alkali-resistant matrix of the holdfast with 3 M hydrochloric acid (HCl) leads to disappearance of the mineral particles, however the fibrous matrix remains stable. In our previously published work, we provided strong evidence that chitin is also resistant to dissolution in HCl [28]. This property can be also effectively used for isolation of chitin that contains calcium carbonate-based minerals as residual material. To confirm this discovery of the presence of chitin within the holdfast of L. baicalensis, we used a multitude of sensitive bioanalytical methods as presented below.

(b) Identification of chitin within holdfast of Lubomirskia baicalensis

Recently, NEXAFS technique has been successfully applied to determine key differences between electronic properties related to the light adsorption by polysaccharides and proteins, even within diverse biominerals [28–30]. We used NEXAFS spectroscopy to explore site-specific electronic properties of L. baicalensis cleaned holdfast samples (measured on 500 × 500 μm areas) in order to gain insight into the nature of the organic components. NEXAFS experiments, performed at the carbon K edge, provided evidence that the contribution of carbon is mainly owing to the organic part of the holdfast (figure 3). Moreover, the carbon K-edge spectrum of this sponge holdfast showed all the typical absorption features of chitin and not those of spongine (figure 3), or collagen, as was the case for spicules of the hexactinellid Hyalonema sieboldi in previous studies by our team [30]. Both chitin and collagen spectra exhibited a strong peak at approximately 288 eV that is associated with the C 1s → π* resonance involving acetamido (−NH(C=O)CH3) group— and pepty (−NH−C(O)−) group—character orbitals. Careful inspection of the spectra indicates, however, that energies of this peak are different for chitin (approx. 288.5 eV), spongine (approx. 288.1 eV) and collagen (approx. 288.2 eV). It has been shown [31,32] that the observed 0.3 eV shift manifests upon the conversion of carboxyl bonds in lonic amino acids into amide bonds in peptide chains. While the 0.3 eV shift is rather small, it has been well documented in the studies cited above. It appears to provide a ‘sensible’ base for ‘in situ’ identification of polysaccharides and proteins—including naturally occurring biocomposites, such as sponge holdfast—without the need of preliminary disruption or extraction. In these analyses, we also show that the C=O-character absorption peak of the holdfast chitin is distinguishable from a strong cellulose peak reported at 289.5 eV (see figure 3; [33]).

The chitin molecule consists of NAG (GlcNAc) residues, including the acetamide group at the C-2 position of glucosamine, the secondary hydroxyl group at C-3 and the primary hydroxyl group at C-6 positions [34]. Therefore, estimation of GlcNAc is the crucial step for chitin identification in organic matrices of unknown origin.
Mass spectroscopy is one of the most sensitive methods for analysis of D-glucosamine, which is the only product of chitin acid hydrolysis. The obtained ESI-MS spectrum of the hydrolyzed *L. baicalensis* holdfast sample is very similar to the spectra of a D-glucosamine standard (see the electronic supplementary material, figure S5), and consists of three main signals with $m/z = 162.18, 180.02$ and 359.61. The signals at $m/z = 180.02$ clearly shows the presence of dGlcN molecules in the sample and corresponds to a $[M + H^+]$ species of dGlcN (calculated molecular weight of 179.1). The signal at $m/z = 162.06$ corresponds to $[M - H_2O + H^+]$ dGlcN ion (calculated: 162.1) which is the loss of one water molecule [35,36]. The weak signal at $m/z = 359.13$ corresponds to $[2M + H^+]$ species which is the proton-bound dGlcN non covalent dimer [36]. The sample at $m/z = 201$ corresponds to $[M - H_2O + K^+]$ and $[(GlcN)_2 + K^+]$ adducts with a potassium ion which is common for natural samples. Interestingly, the sample can be completely hydrolyzed at 60°C, but is stable in 6 M HCl at room temperature for at least 24 h.

To quantify chitin in our samples, we measured the amount of N-acetylglucosamine released by chitinases using a Morgan–Elson colorimetric assay [37], which is the most reliable method for the identification of alkali-insoluble chitin owing to its specificity [38]. We detected 775.3 ± 0.3 µg N-acetyl-glucosamine per mg of *L. baicalensis* holdfast.

The results of Raman spectroscopy of the cleaned *L. baicalensis* holdfast are represented in the electronic supplementary material, figure S6. The Raman spectra for α-chitin and *L. baicalensis* cleaned fibrous holdfast fibre material were nearly identical, also in agreement with published reports on chitin identification using Raman spectroscopy [39].

In summary, the analytical investigations described above clearly show the presence of chitin within the holdfast of *L. baicalensis*. This observation raises the question of the presence of the corresponding CHS genes, which have thus far not been described. We have, therefore, carried out the corresponding genetic analysis, as described below.

**c) Characterization of chitin synthase genes in *Lubomirskia baicalensis***

We have isolated and characterized four new CHS gene fragments from the freshwater sponge *L. baicalensis*: CHS_LB01 (1088 bp), CHS_LB02 (924 bp), CHS_LB03 (1077 bp) and CHS_LB04 (1232 bp). The first three sequences were identified from sponge RNA, while CHS_LB04—from sponge DNA. These sequences included the intron in position 935–1073 (139 bp). The sizes of the deduced hypothetical protein fragments of Baikalian sponge CHSs were 308–364 aa. A comparison of CHS amino acid sequences yielded an identity of 74.5–92.5 per cent. BLAST-X analyses indicated that the protein sequences of CHS from Baikalian sponge were most similar to CHS of *S. lacustris*, AEI55440 (73–98%), *A. queenslandica*, XP_003385441 (52–54%), *Hydra magnipapillata*, XP_001262504 (39–43%), *Nematostella vectensis*, XP_001633545 (43–46%) and *Branchiostoma floridae*, XP_002592717 (35–39%). CLUSTAL alignment of *L. baicalensis* CHS predicted proteins with those from the closest relatives revealed the conserved domain specific for all types of CHSs. This domain includes catalytically critical sequences GEDR and QRRRW [40] in the all amino acid sequences (figure 4).

**4. Discussion**

The presence of chitin in both evolutionary older marine and evolutionary younger freshwater sponges [42] suggests that the CHS genes found in this study represent shared ancestral character states of sponges, and maybe even of a possible common ancestor within the metazoan lineage (see the electronic supplementary material, figure S7). Thus, we suggest that the chemistry, structure, morphology and biomechanical properties of poriferan holdfasts were crucial throughout the evolutionary history of sponges. Since dislodgment is mostly fatal for adult sponges, the role of the holdfast is a critical one. For sponges, holdfast morphology and sediment cohesive-ness are important determinants of the maximum tensile force they are able to withstand under specific environmental conditions.

There are no doubts that fixation is very important for all sedimentary animals, especially those which have little opportunity to move or to re-build these body parts. Intriguingly, to our best knowledge, there are no reports regarding the chitinous origin of the holdfast of other aquatic invertebrates with exception of hydrorhiza of the hyroid *Myriotheca cocksi* [43]. Hydrorhiza is a rootstock by which a hyroid is attached to other objects. The adhesion of the hydrorhiza to the substratum is affected only by the perisarc layer covering the flattened extremities of the adhesive tentacles. This perisarc is about 6 µm thick and is composed of true chitin [43].

The comparably smaller sponge-class Calcarea is characterized by skeletal spicules of calcite, and holdfasts within this taxon are less expressive, than those found among members of the class Demospongiae, which are notably more diverse, reach large sizes and inhabit more different solid substrata (for review see [8]). Searching for specific types of fixation including some hypothetical adhesive substances for fixation in different classes of Porifera is a challenging task for future studies.
Within the phylum Porifera, different attachment methods have evolved over time. For example, the settled larvae of *Halichondria moorei* undergoing metamorphosis were found to possess a complex glycocalyx lining the cells on their upper surface [44]. This structure, which has been referred to as the sponge larval coat, was present on neither adult sponges, nor on unsettled larvae. It was suggested that this sponge's mechanism for larval attachment bears some similarity to the adhesion of many cultured cells to their substrates. This hypothesis is supported by the absence in sponge larvae of specialized cement glands, which are known to be involved in substrate attachment in other marine invertebrates [44].

Our finding of the presence of chitin within holdfast of *L. baikalensis* suggests the existence of corresponding chitin-producing cells. Intriguingly, these cells are definitively not located within chitin-containing gemmules. In numerous freshwater sponges, gemmules consist of a rough coating (mainly proteinaceous spongina and silicious gemmuloscleres) and are filled with archaeocytic totipotent cells and many undifferentiated and polynucleated cells [45]. It was found that newly formed gemmular cells are first surrounded by a squamous cell layer which later degenerates and becomes surrounded in turn by a layer of columnar cells. The columnar cells secrete both internal and external chitinous membranes. Jeuniaux [46] reported that 1–3% of the dry weight of the gemmules’ coating is chitin. However, while the production of dormant, encapsulated gemmules by freshwater sponges and a few marine species is a well-documented biological phenomenon [47], there are to date no reports about gemmule production by any species of the endemic family Lubomirskiidae, freshwater sponges in the Baikal Lake, which is monophyletic with the globally distributed Spongillidae [48]. Thus, an obvious object of investigation with respect to identification of chitin is the poorly investigated larva of *L. baikalensis*.

One of the most intriguing questions that shall be addressed by further investigations is to decipher the role of the holdfast in sponge attachment, and how holdfasts maintain their adhesive prosperities underwater and in highly saline as well as in freshwater conditions. Such information may have a high potential for use in biomimetic fields, where it could be exploited for the design of biocompatible adhesives with tuneable physico-chemical properties.

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