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Three-dimensional chitin-based scaffolds from Verongida sponges (Demospongiae: Porifera). Part I. Isolation and identification of chitin

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ABSTRACT

Marine invertebrate organisms including sponges (Porifera) not only provide an abundant source of biologically active secondary metabolites but also inspire investigations to develop biomimetic composites, scaffolds and templates for practical use in materials science, biomedicine and tissue engineering. Here, we presented a detailed study of the structural and physico-chemical properties of three-dimensional skeletal scaffolds of the marine sponges *Aiolochroia crassa*, *Aplysina aerophoba*, *A. cauliformis*, *A. cavernicola*, and *A. fulva* (Verongida: Demospongiae). We show that these fibrous scaffolds have a multilayered design and are made of chitin. ^{13}C solid-state NMR spectroscopy, NEXAFS, and IR spectroscopy as well as chitinase digestion and test were applied in order to unequivocally prove the existence of α-chitin in all investigated species.

1. Introduction

The formation of extended skeletal structures often involves hierarchical processing: assemblies of organic molecules are used to build the basic framework blocks which pre-determine the organization of subsequently produced inorganic deposits. The resulting inorganic materials may in turn be used as building units for the production of more complex structures of higher order [1]. Animal tissues use a variety of skeletal structures. The two most abundant systems make use of collagen or chitin [2] as major framework constituents. The collagenous system is based on the association between collagen – a unique fibrous protein – and varying quantities of non-collagenous proteins. In the chitin system, the aminopolysaccharide chitin is combined with non-collagenous proteins. Both, collagen and chitin are extracellular secretions, usually with a conspicuous fibrous organization at different hierarchical levels (nanofibrils–microfibrils–fibers). Likewise, collagen

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and chitin structures may serve as scaffolds for amorphous or/and crystalline inorganic deposits. The chitin systems are usually of ectodermal origin. Chitin is involved in exoskeleton formation. In contrast, the collagen systems are almost exclusively of mesodermal origin and are involved in endoskeletons. Phylogenetic studies suggest that the chitin systems of fungi and animals are related [3,4] and appeared before collagen systems evolved. Because of their different developmental and evolutionary origin, the chitin and the collagen system are usually considered to be independent [2]. However, we have recently discovered that chitin is incorporated into the spongin-based skeletal fibers of the sponges (Phylum Porifera) *Verongula gigantea* and *Ianthella basta* which belong to the order Verongida (class Demospongiae) [5–7]. There are 14 taxonomic orders within the class Demospongiae. This order encompasses about 95% of extant sponges. Three of these orders – Verongida, Dictyoceratida, and Dendroceratida – exhibit skeletons without siliceous spicules. Instead, the skeletons consist of spongin fiber networks. Spongin is a protein resulting from a super-compaction of collagen fibrils and filaments [8–10]. Recent phylogenetic and embryological studies have shown that Verongida, Dictyoceratida, and Dendroceratida – although being characterized by fibrous spongin skeletons – do not make up a cohesive phylogenetic unit [11,12]. The recently discovered [5–7] chitin/collagen composite fiber skeletons found in the aforementioned Verongida sponge species have apparently evolved independently from Dictyoceratida and Dendroceratida [12].

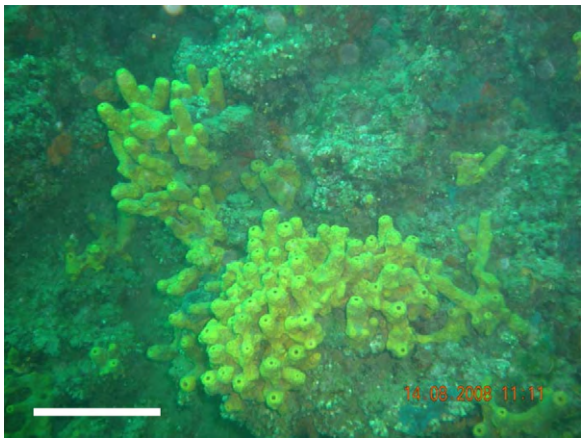

Fig. 1. *Aplysina aerophoba* – a typical representative of Verongida sponges, in its natural underwater environment (Kotor Bay, Montenegro, scale bar: 50 cm).

However, a systematic study of the numerous other species belonging to the order Verongida has not yet been performed. Sponges are probably the earliest branching animals with a fossil record dating back to the Precambrian [13,14]. This is the reason why the presence of chitin in sponges is also of evolutionary interest. But the elucidation of the three-dimensional organization of

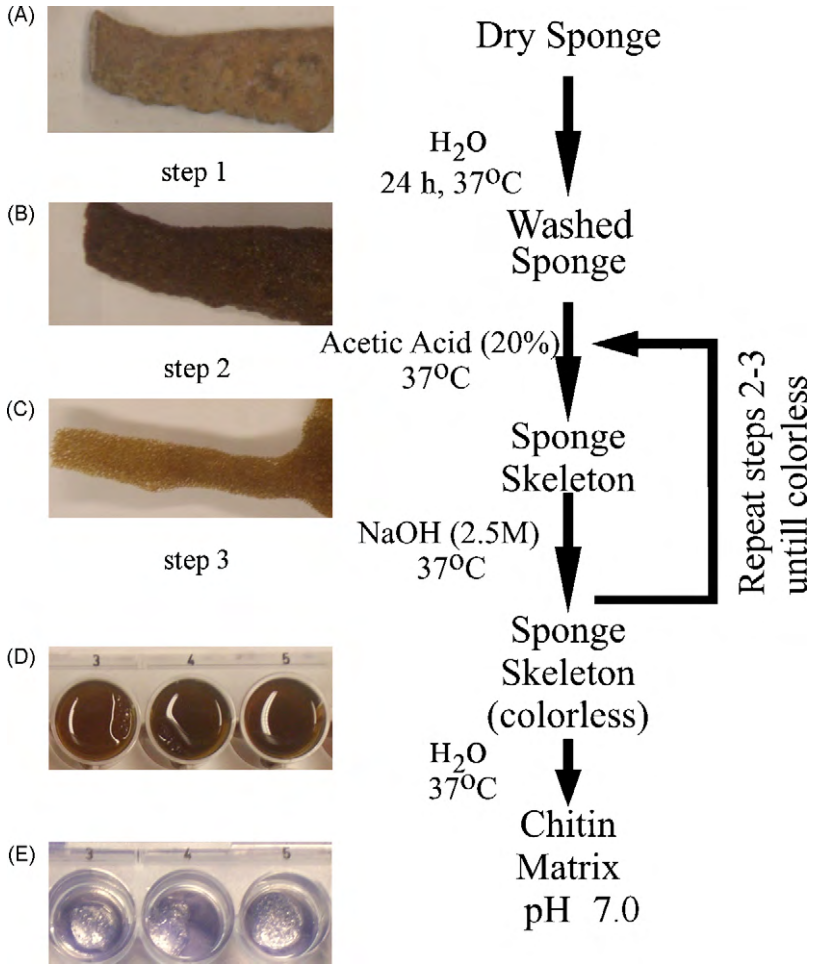

Fig. 2. Scheme of the isolation process of the chitin-based scaffolds of Verongida sponges. The images demonstrate the result of the corresponding treatment steps for the example of *A. cauliformis*. The images show an *A. cauliformis* sample after extraction steps 1 (A), 2 (B), and 3 (C, NaOH treatment not repeated) as well as the extract solution after step 3 (D) and a colorless scaffold sample (E) obtained after finishing the extraction procedure.

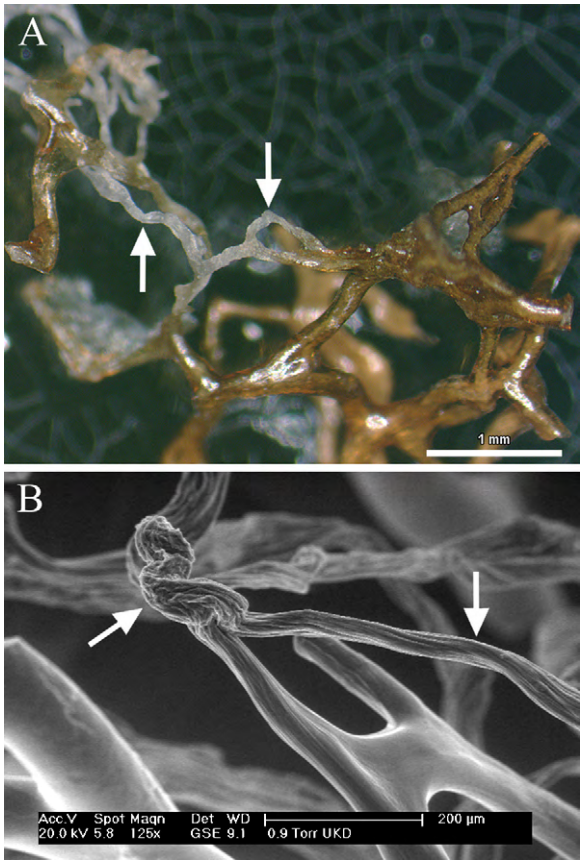

Fig. 3. Light microscopic (A) as well as SEM (B) images clearly show differences in pigmentation and structural integrity between natural and selectively demineralized parts of the skeletal network of *A. aerophoba*.

their chitin structures may not only be of biological/evolutionary interest.

The goals of the present paper was to carry out systematic screening for the presence of chitin-based scaffolds within the Aplysinidae family, order Verongida ant to confirm their chitinous nature. The following representatives of this family were investigated: *Aiolochroia crassa*, *Aplysina aerophoba*, *A. cauliformis*, *A. cavernicola*, and *A. fulva*. The samples were obtained from different locations (see Section 2).

2. Materials and methods

2.1. Sample preparation

A. cavernicola was collected from the Mediterranean Sea by SCUBA diving from a population located between 14 and 16 m depth on a vertical, calcareous rocky wall substrate in Maire Island (Marseille, France). *A. cauliformis*, *A. fulva*, and *A. crassa* were collected from the Caribbean Sea from a well illuminated coral reef located in the eastern part of Grand Bahama Island (Sweetings Cay, Bahamas) between at 4 and 8 m depth. *A. aerophoba* was collected in the Adriatic Sea (Kotor Bay, Montenegro) (Fig. 1).

Sponge samples were put in ziplock bags underwater, brought back to the laboratory and frozen less than an hour after collection. The sponges were lyophilized prior to further treatment. The use of chemicals was avoided in the cleaning and preparing procedure of the skeletons.

2.2. Isolation of chitin-based scaffolds from Verongida sponges

Chitin has been extracted from the sponges by subjecting them to the chemical treatment specified below [15,16]. To remove other compounds from the chitin, the sample underwent a series of extraction steps. Each was designed to remove impurities having different properties. These extractions included step-by-step treatment as follows: an acidic extraction, an alkali-based extraction, an optional hydrogen peroxide treatment, and washing steps using distilled water before and after each treatment step.

Step 1: The samples were washed with distilled water at 37 °C for 24 h. This resulted in the extraction of all water-soluble substances including several pigments. Lysis of the sponge cells was also caused by this step of the treatment.
Step 2: Acidic extraction at 37 °C involved sample treatment with an acid solution in order to degrade possible calcium carbonate containing constituents and to remove acid-soluble proteins and pigments [15,16]. The samples were treated in 20% acetic acid under stirring for 24 h. The remaining three-dimensional fibrous sponge skeleton was neutralized and subjected to further treatment steps.
Step 3: Alkali-based extraction at 37 °C involved sample treatment with a solution of 2.5 M NaOH in order to degrade and remove the sponge lipids and proteins as well as to eventually remove residual silica and pigments. Alkali treatment was performed for 24 h under stirring. The remaining three-dimensional scaffolds consisting of a fibrous skeletal material were neutralized. The procedure listed above was repeated until a colorless fibrous material remained (Fig. 2).

Hydrogen peroxide (35%) treatment can optionally be performed at room temperature under stirring for 15 min in order to degrade residual pigments. After H_2O_2 treatment, the residual three-dimensional fibrous sponge skeletal material was washed using distilled water and stored at 4 °C.

2.3. ^{13}C solid-state NMR spectroscopy

Solid-state ^{13}C NMR experiments were performed on a Bruker Avance 300 spectrometer operating at 75.47 MHz for ^{13}C using a commercial double resonance 4 mm MAS NMR probe. Ramped ^{1}H–^{13}C cross-polarization [17,18] was used (contact time: 4 ms). SPINAL ^{1}H-decoupling [19] was applied during the signal acquisition. The spectra were referenced relative to tetramethylsilane (TMS).

2.4. NEXAFS spectroscopy

The electronic structure of demineralized fibrous scaffolds isolated from the investigated Verongida sponges was characterized by near-edge X-ray absorption fine structure (NEXAFS) spectroscopy at the BESSY (Berlin) as described previously [20].

2.5. Scanning electron microscopy (SEM) analysis

The samples were fixed in a sample holder and covered with carbon for 1 min using an Edwards S150B sputter coater. The samples were then placed in an ESEM XL 30 Philips or LEO DSM 982 Gemini scanning electron microscope.

2.6. FTIR spectroscopy

Infrared spectra were recorded on a Nicolet 210c FTIR Spectrometer. The samples were embedded in KBr and measured in

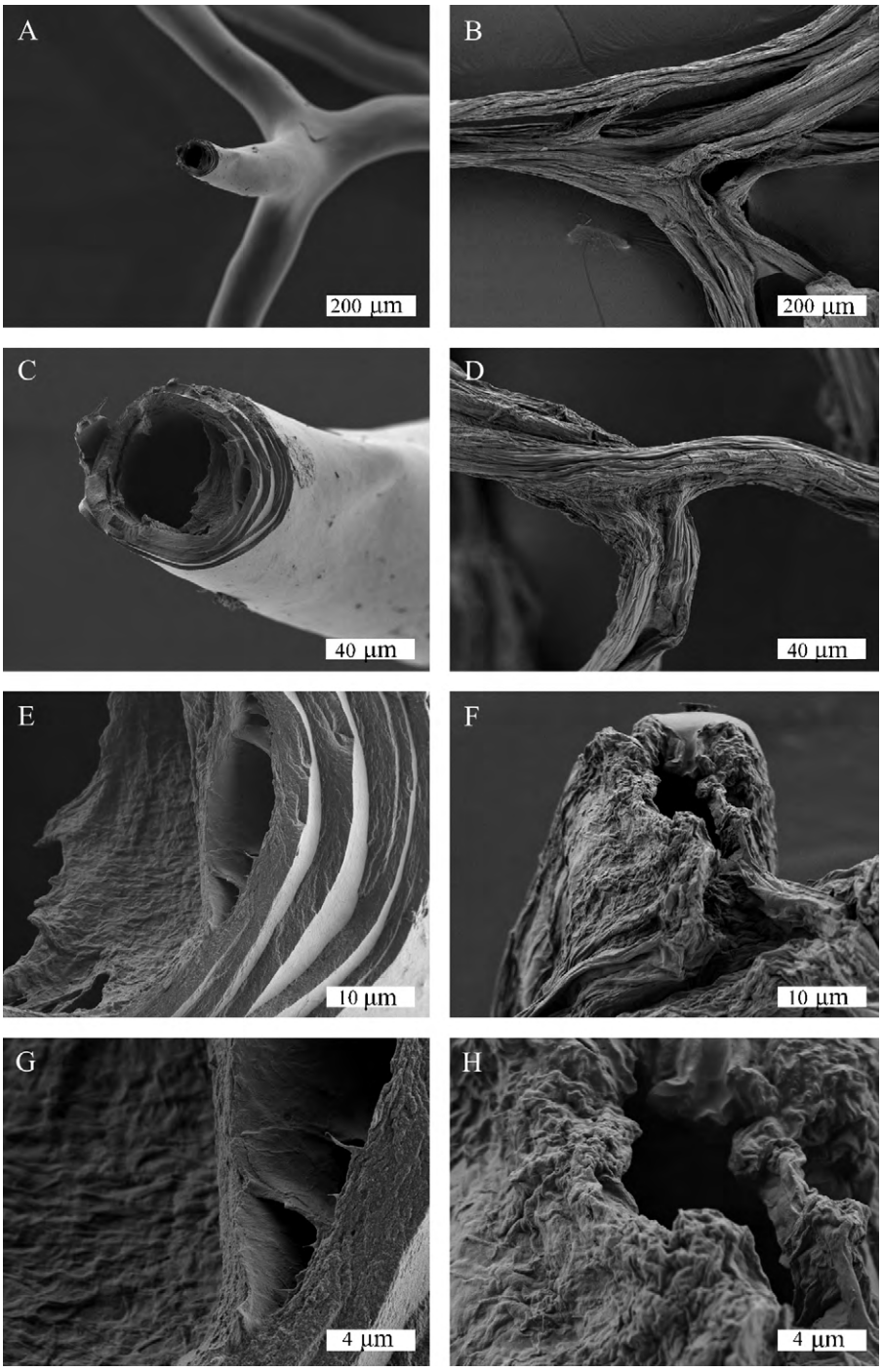

Fig. 4. Left: SEM images of untreated *A. aerophoba* fibers (A, C, E, G). Right: alkali-treated fibers (B, D, F, H). The shape of demineralized and depigmented fibers closely resembles the shape of the sponge skeleton. Comparable results were obtained for all of the investigated Verongida sponges.

transmission. The spectral resolution of the spectra amounts to 2 cm^{-1}.

2.7. Chitinase digestion and test

Chitinase (EC 3.2.1.14, No. C-8241, Sigma) from the fungus *Trichoderma viride* was used. One unit of this chitinase releases 1.0 mg of N-acetyl-D-glucosamine from chitin per hour at pH 6.0 and 25 °C. Fiber portions of selected Verongida sponges (8.0 mg) were incubated in chitinase, dissolved in 0.2 M citrate phosphate buffer at pH 4.5 at 25 °C for 12 h. Enzyme solutions were made in the same buffer at a concentration of 0.5 mg/mL. Different reaction times up to 12 h were used as specified in text. The effectiveness of the enzymatic digestion was monitored using SEM and optical microscopy (Zeiss, Axiovert).

2.8. Estimation of N-acetyl-D-glucosamine (NAG) contents

Preparation of colloidal chitin from a crab α-chitin standard (Sigma) was performed according to Boden et al. [21]. The Morgan-Elson assay was used to quantify the N-acetyl-D-glucosamine released after chitinase treatment as described previously [21].

Dried sponge skeleton samples (6 mg) were pulverized to a fine powder in an agate mortar. The samples were suspended

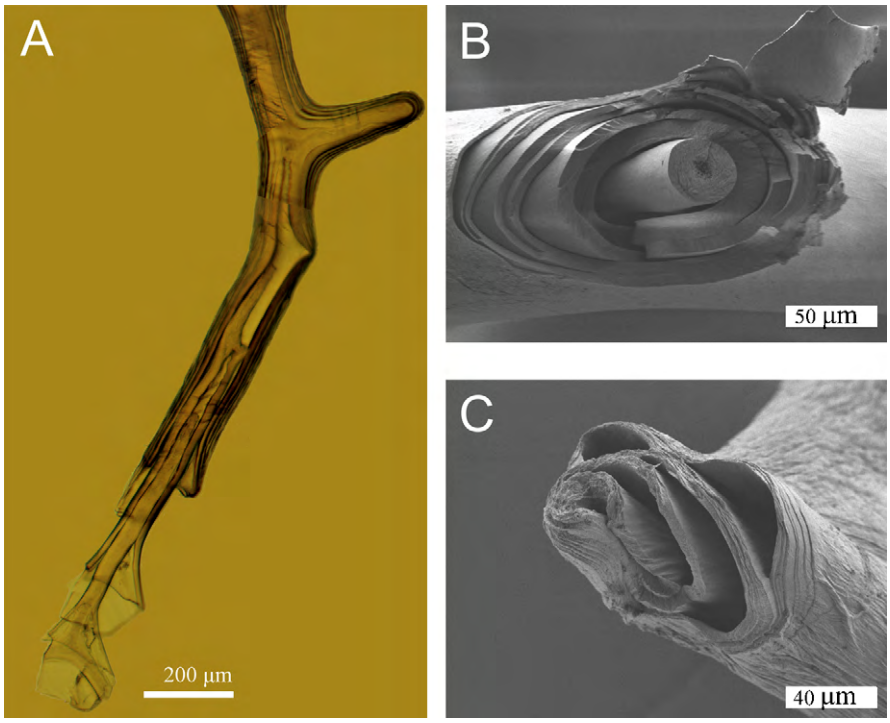

Fig. 5. Multilayered structures of the verongid spongin fibers. (A) Light microscopy and (B, C) SEM images reveal the layered structures. Numerous layers are visible in an *A. aerophoba* demineralized fiber (A) which was stretched out using forceps, as well as in the cross-section cut natural (B) and demineralized (C) fiber of *A. cauliformis*.

in 400 ml of 0.2 M phosphate buffer at pH 6.5. Positive control was prepared by solubilizing 0.3% colloidal chitin [6] in the same buffer. Equal amounts of 1 mg/mL of three chitinases (EC 3.2.1.14 and EC 3.2.1.30): N-acetyl-D-glucosaminidase from *T. viride* (Sigma, No. C-8241), and two poly(1,4-β-[2-acetamido-2-deoxy-D-glucoside]) glycanohydrolases from *Serratia marcescens* (Sigma, No. C-7809) and *Streptomyces griseus* (Sigma, No. C-6137) respectively, were suspended in 100 mM sodium phosphate buffer at pH 6.0.

Digestion was started by mixing 400 ml of the samples and 400 ml of the chitinase mix. Incubation was performed at 37 °C and stopped after 114 h by adding 400 ml of 1% NaOH, followed by boiling for 5 min. The vessels were centrifuged at 7000 rpm for 5 min and the produced reducing sugars were determined using the 3,5-dinitrosalicylic acid assay (DNS) [22]. For this purpose, 250 ml of the supernatants and 250 ml of 1% DNS were dissolved in a solution containing 30% sodium potassium tartrate in 0.4 M NaOH, mixed and incubated for 5 min in a boiling water bath. Thereafter, the absorbance at 540 nm was recorded using a Tecan Spectrafluor Plus Instrument (Mannedorf/Zurich, Switzerland). Data were interpolated in a standard curve prepared with a series of dilutions (0–3.0 mM) of N-acetyl-D-glucosamine (Sigma, No. A-8625) and DNS. The sample which contained chitinase solution without substrate was used as control.

3. Results and discussion

3.1. Isolation and demineralization of skeletal networks

Verongida sponges are characterized by spongin fibers which are strongly and regularly anastomosed (Fig. 3) thus forming skeletal networks. Sometimes, these networks are compressed into two dimensions. The fibers have a central pith and a laminated peripheral bark. The latter may contain cellular elements in concentric annuli. Occasionally some of the cells may occur scattered throughout the pith. The fiber skeleton makes up the major bulk of the sponge. Fragments of the sponges were treated in distilled water, acetic acid, sodium hydroxide and optionally hydrogen peroxide as described in Section 2. Fibers incorporating chitin should be more resistant against alkali solutions (Fig. 3) than those consisting of spongin [23] only. The dissolved pigmented fraction was isolated by centrifugation (see Fig. 2D). The residual (skeletal) fraction was dialyzed, air dried and analyzed by various methods (SEM, ^{13}C solid-state NMR spectroscopy, NEXAFS spectroscopy, IR and

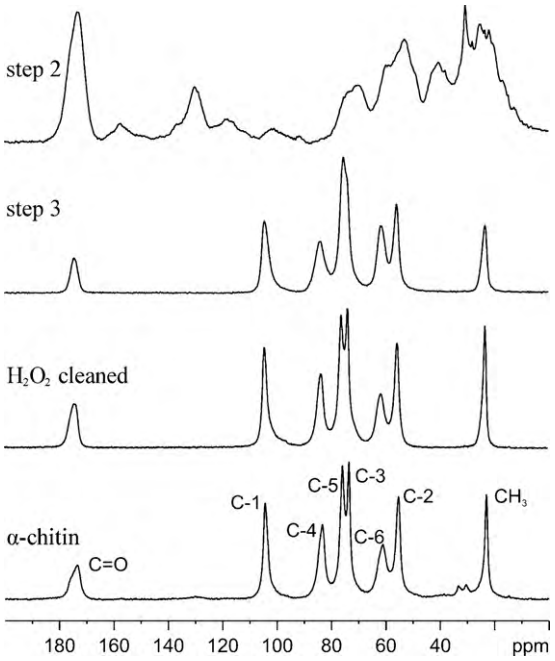

Fig. 6. ^{13}C {^{1}H} CP MAS NMR spectra (293 K) of *A. cauliformis* skeleton samples measured after the different isolation steps (see Fig. 2). The spectrum of the α-chitin control sample is shown for comparison.

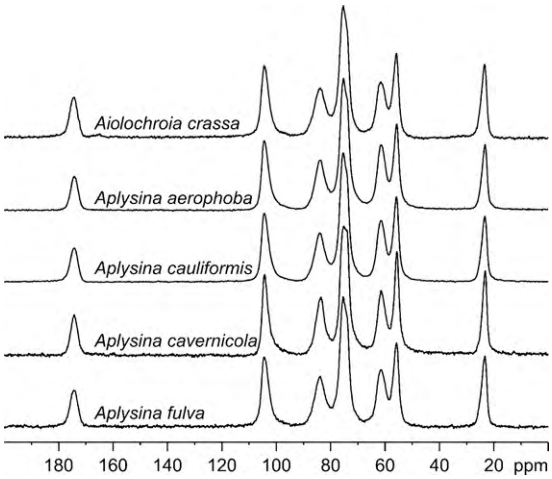

Fig. 7. ^{13}C $\{^{1}H\}$ CP MAS NMR spectra (293 K) of the different Verongida sponge samples measured after NaOH treatment (see Fig. 2, step 3).

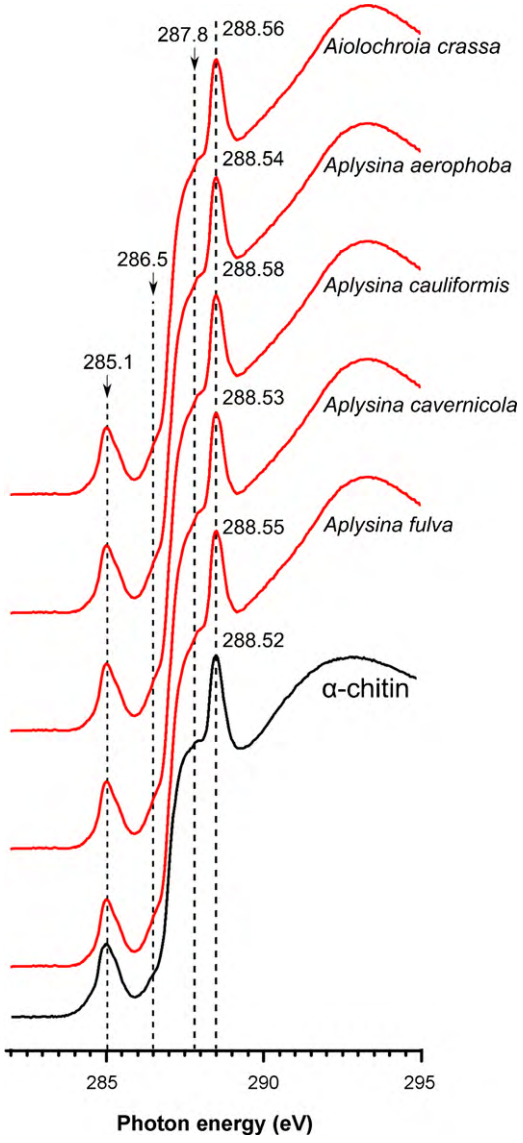

Fig. 8. NEXAFS spectra of the organic scaffolds isolated from the investigated Verongida sponges and the α-chitin standard taken at the C 1s threshold. The spectra of the extracted scaffolds clearly agree with the α-chitin standard indicating the chitinous nature of the extracted scaffolds after treatment step 3.

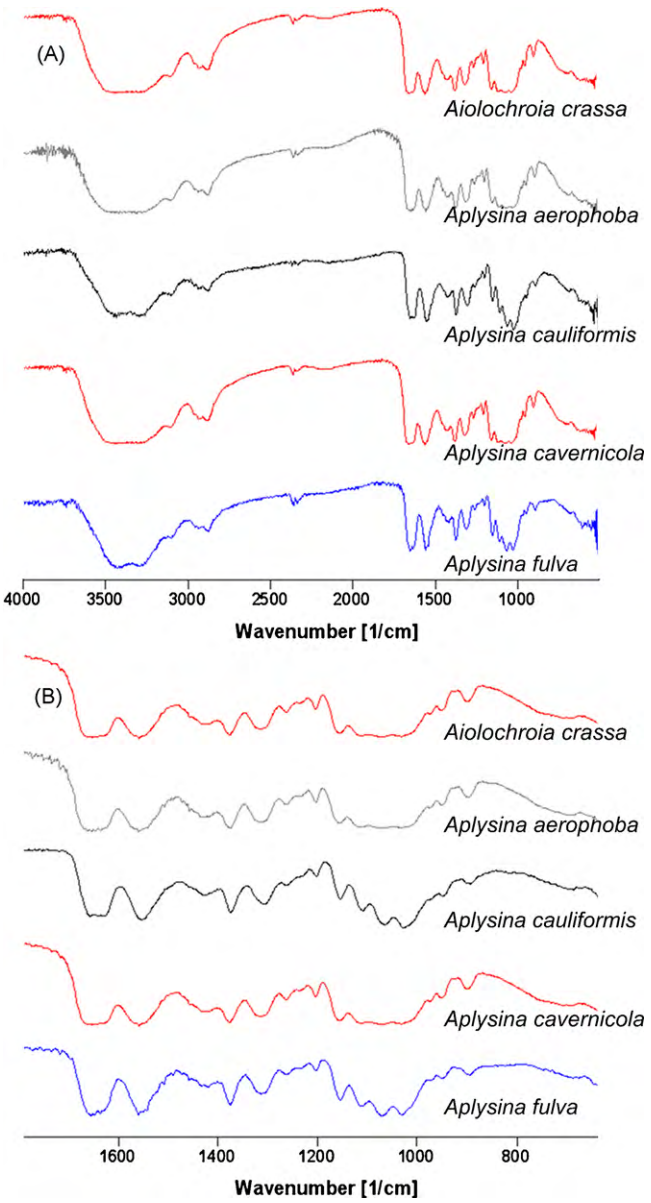

Fig. 9. FTIR spectra of NaOH treated (step 3) of Verongida sponge samples. (A) "A" shows a larger wavenumber range than "B". The spectra again prove the existence of chitin in the verongid skeletal formations. The obtained spectra are very similar to those reported previously, for band assignment see [6,7].

Raman spectroscopy, fluorescence microscopy) as well as biochemical approaches (chitinase digestion and test).

First of all, it can be stated that the overall shape and morphology of the extracted skeletons closely resemble the original shape and morphology of the sponges under study. That means the extraction procedure does not lead to a breakdown of the – sometimes very delicate – sponge structures. A closer look however shows that the alkali treatment leads to characteristic partial deformations (wrinkling) of the skeletal fibers, probably due to the extraction of stabilizing substances such as spongin and others (Figs. 3 and 4).

This suggests that at least some of the extracted compounds act as cross-linking agents. The removed compounds may also provide the fibers with an extended resistance against natural degradation (e.g. by bacterial/chemical attack, mechanical abrasion, etc.). In other organisms, chitin occurs associated with various types of proteins, polysaccharides, and minerals (usually calcium and magnesium carbonates), as well as lipids and pigments. The compo-

sition is characteristic for the individual organism. The interaction between chitin and the other molecules/phases is often the key for the mechanical stability [24] of the specific biomaterial.

At the end of the treatment procedure, the fibers exhibit a hollow, pipe-like, and translucent structure. Selective demineralization and depigmentation of the skeletal network by partial immersion of the skeletal fibers into the alkali solution nicely demonstrates the influence of the extraction procedure (Fig. 3A and B).

Light microscopy (Fig. 5A) as well as SEM observations (Fig. 5B and C) of the skeletal fibers confirmed the characteristic organization of the sponge fibers into concentric layers. Cross-sections of the fibers revealed distinct concentric layers. These layers were still visible after demineralization (Fig. 5C). This "cylinder-within a cylinder" construction is similar to the structure of the siliceous spicules found in hexactinellid sponges [25–27].

3.2. Identification of chitin

The analytical characterization included ^{13}C solid-state NMR spectroscopy (Figs. 6 and 7), NEXAFS (Fig. 8), and FTIR spectroscopy (Fig. 9). These methods consistently revealed that the alkali-resistant, fibrous material remaining after demineralization consists of α-chitin for all species under study.

3.3. Chitinase digestion

Verongida sponges contain enormous amounts of symbiotic as well as opportunistic bacteria and cyanobacteria within their bodies (see, e.g. [12,28]). It cannot be excluded that extracellular enzymes such as proteases, collagenases, or/and chitinases produced by either the internal microbial populations or opportunistic foreign microbes entering through mechanical lesions of the sponge ectosome could attack the integrity of the spongin skeleton. Bacterial attack of spongin fibers associated with several sponge diseases has been described previously [28–31]. It has been suggested that skeletal fibers of verongids incorporate brominated compounds (e.g., bromotyrosines [32,33]), which might have an antibacterial effect. Jaspars and co-workers [34] reported that brominated tyrosine-derived compounds isolated from the Verongida sponge *Aplysinella rhax* moderately inhibited bacterial chitinase. To examine the level at which chitin protects spongin fibers from bacterial attack, we treated natural chitin-bearing fibers of the verongid *I. basta* and chitin-based networks from several other origins using commercial chitinase from *T. viride* [7]. After 24 h of incubation with chitinase, no visible changes were noticed in *I. basta* fibers when studied under both light microscopy and SEM. We also treated fibers of *V. gigantea* and the *Aplysina* species studied here and obtained comparable results (data not shown). However, NaOH-extracted chitin-based scaffolds were readily digested by the chitinase solution even during the first 6 h. This treatment resulted in the release of residual chitin microparticles of steadily decreasing size (Fig. 10).

Chitinase treatment also leads to the visualization of the nanofibrillar organization of the chitin-based scaffolds, especially during the initial stages of digestion (Fig. 11).

It is known that chitin is insoluble in most solvents due to its specific structure which is based on hydrogen bonding among acetamide groups, hydroxyl groups, and carbonyl groups [35]. The chitin molecule consists of N-acetyl-D-glucosamine (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 [36]. Chitin oligomers of more than 10 monomers are hardly soluble in water and spontaneously assemble into fibers [37].

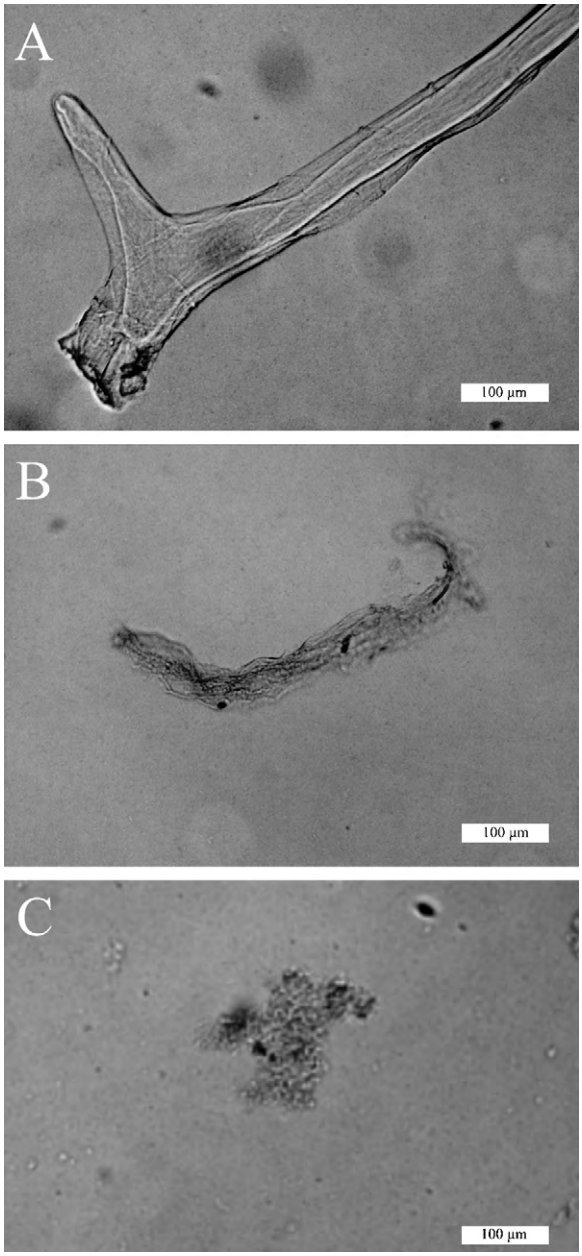

Fig. 10. Chitinase digestion of a NaOH-extracted and H_2O_2 purified scaffold fragment from *A. aerophoba* (light microscopic images). A – initial stage; B, and C – after 4 h and 6 h of chitinase treatment, respectively.

Hierarchical organization is characteristic for numerous biological materials based on chitin. In all cases, the first level of the organization of these materials is the chitin chain along the *c*-axis where hydrogen ions are laterally spaced by 0.475 nm with a monomer length of 1.032 nm. The second level is made by nanofibrils of about 2–3 nm in diameter and about 300 nm in length, each containing 19 chains. The number of chitin chains in the nanofibril is probably close to a minimum for stability. Hence, the chitin nanofibrils present an optimum surface area for interfacial interactions within corresponding structural formations [38]. The lateral nanofibrillar dimensions can range from 2.5 to 25 nm (Fig. 11D), depending upon the organism [39]. The third level consists of microfibrils. They occur as shallow helices which may be right- or left-handed [24]. The fourth level is made by "fibers" of more than 1 µm in diameter [40] (Fig. 10B). In some cases, there is a diversity of chitin aggregation within one and the same organism.

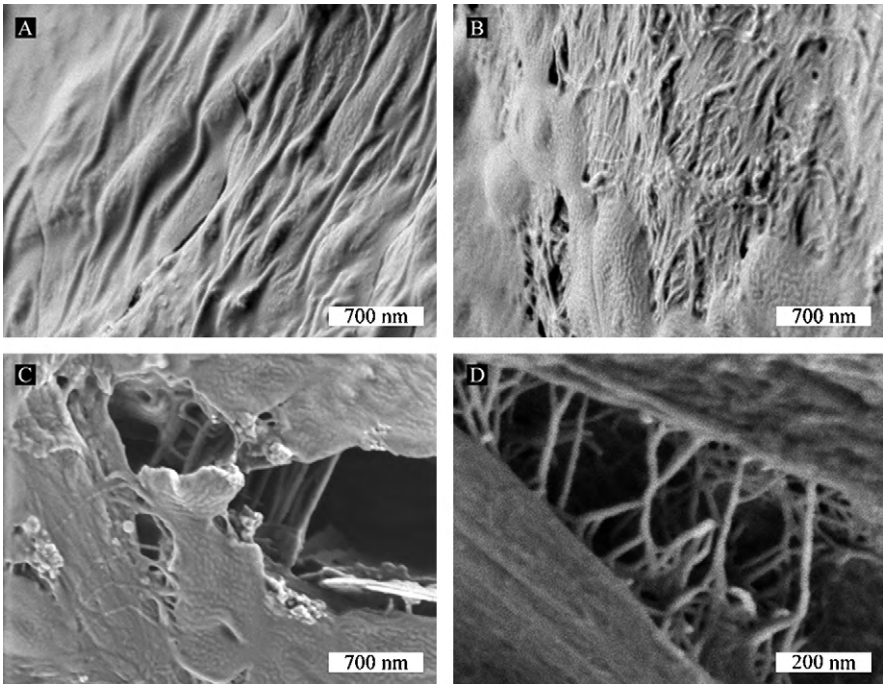

Fig. 11. SEM observation of the chitinase activity during the first hour of enzymatic treatment with respect to the visualization of the nanostructural organization of a NaOH-extracted *A. aerophoba* scaffold. A – prior to treatment; B – after 20 min; C and D – after 1 h.

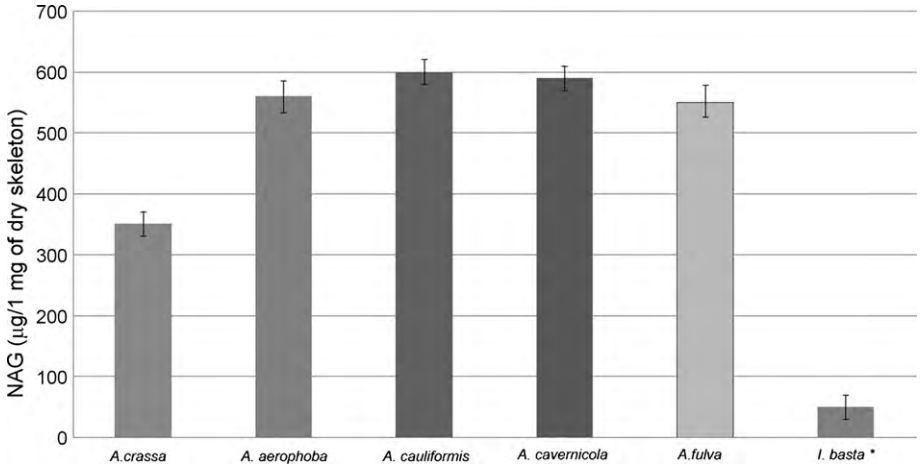

Fig. 12. Results of the Morgan-Elson assay for the determination of N-acetyl-D-glucosamine (NAG) after 114 h of insertion in chitinase solution. Data for *I. basta* were obtained previously [7].

For example, chitin appears in three different organs in shell-free eolid nudibranchs (Mollusca): (1) in the radular teeth; (2) in cuticles of the head alimentary tract; and (3) as intracellular granules in the epidermal cells of the skin and the gut epithelium, known as the spindles [41]. Despite the structural similarity at the nano- and microlevel between the chitin of sponges and that of other invertebrates, the pathways of the chitin biosynthesis as well as the principles of the chitin nano- and microfibril assembly in sponges are unknown yet.

The Morgan-Elson assay for the determination of N-acetyl-D-glucosamine (NAG) in chitin-based scaffolds indicated some variability among the various verongid species studied (Fig. 12). The measured NAG concentrations fall within the range of those previously measured in the fibers of *V. gigantea* [6]. No measurable NAG concentrations were found in experiments where the chitinase treatment was performed on natural (i.e. non-extracted) skeletal fibers of the sponges which still contain the aforementioned bromotyrosine-related and other compounds. Therefore, at least one of these substances seems to be responsible for preserving chitin-containing spongin fibers against enzymatic degradation in the marine environment.

4. Conclusions

The present systematic study of representatives from the Aplysinidae family revealed the presence of a chitin-based scaffold closely resembling the shape and morphology of the original sponge for all species under study. Taken together with our previously published data concerning the species *V. gigantea* and *I. basta*, we have now good reason to assume that such chitin-based scaffolds are characteristic for the order Verongida and not just for a small number of singular species. The possible chitin biosynthesis pathways in Verongida sponges remain to be a challenge for

future research. The results of investigations of potential of three-dimensional chitin-based scaffolds with respect to application in tissue engineering are reported in the second part of this paper.

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