

First Evidence of Chitin as a Component of the Skeletal Fibers of Marine Sponges. Part I. Verongidae (Demospongia: Porifera)

HERMANN EHRLICH^{1*}, MANUEL MALDONADO²,
KLAUS-DIETER SPINDLER³, CARSTEN ECKERT⁴, THOMAS HANKE¹,
RENÉ BORN¹, CAREN GOEBEL⁵, PAUL SIMON⁵, SASCHA HEINEMANN¹,
AND HARTMUT WORCH¹

¹Max Bergmann Center of Biomaterials, Institute of Materials Science,
Dresden University of Technology, D-01069 Dresden, Germany

²Centro de Estudios Avanzados de Blanes (CSIC), 17300 Blanes Girona, Spain

³Department of General Zoology and Endocrinology, University of Ulm,
D-89069 Ulm, Germany

⁴Museum für Naturkunde, Zentralinstitut der Humboldt-Universität zu Berlin,
Institut für Systematische Zoologie, D-10115 Berlin, Germany

⁵Max Planck Institute of Chemical Physics of Solids, D-01187 Dresden, Germany

ABSTRACT The Porifera (sponges) are often regarded as the oldest, extant metazoan phylum, also bearing the ancestral stage for most features occurring in higher animals. The absence of chitin in sponges, except for the wall of peculiar resistance bodies produced by a highly derived fresh-water group, is puzzling, since it points out chitin to be an autapomorphy for a particular sponge family rather than the ancestral condition within the metazoan lineage. By investigating the internal proteinaceous (spongin) skeleton of two demosponges (*Aplysina* sp. and *Verongula gigantea*) using a wide array of techniques (Fourier transform infrared (FTIR), Raman, X-ray, Calcofluor White Staining, Immunolabeling, and chitinase test), we show that chitin is a component of the outermost layer (cuticle) of the skeletal fibers of these demosponges. FTIR and Raman spectra, as well as X-ray diffractograms consistently revealed that sponge chitin is much closer to the α -chitin known from other animals than to β -chitin. These findings support the view that the occurrence of a chitin-producing system is the ancestral condition in Metazoa, and that the α -chitin is the primitive form in animals. *J. Exp. Zool. (Mol. Dev. Evol.)* 308B:347–356, 2007. © 2007 Wiley-Liss, Inc.

How to cite this article: Ehrlich H, Maldonado M, Spindler K-D, Eckert C, Hanke T, Born R, Goebel C, Simon P, Heinemann S, Worch H. 2007. First evidence of chitin as a component of the skeletal fibers of marine sponges. Part I. verongidae (demospongia: porifera). *J. Exp. Zool. (Mol. Dev. Evol.)* 308B:347–356.

Chitin is, after cellulose, the most abundant polysaccharide in nature, usually complexed with other polysaccharides and proteins. It is known to occur as a component of the cell wall in fungi and diatoms (Herth and Zugenmaier, '77; Nguyen et al., '98; Peter, 2002), also in diverse structures of at least 19 animal phyla (Willmer, '90). The distribution of chitin among the animals appears to support the hypothesis that chitin-coding gene(s) were lost at the root of the deuterostome lineage (Rudall, '55; Jeuniaux, '63; Rudall and Kenchington, '73; Wagner, '94), since chitin occurs mostly in diploblasts and triploblastic protostomes (14 phyla),

being less common among the deuterostomes (five phyla).

The question whether chitin is present in sponges has been poorly studied until now. It was reported that the presence of chitin in some sponge macerates is actually due to a variety of

*Correspondence to: H. Ehrlich, Max Bergmann Center of Biomaterials, Dresden University of Technology, Budapest Str. 27, 01069 Dresden, Germany.
E-mail: hermann.ehrlich@mailbox.tu-dresden.de

Received 12 October 2006; Revised 20 December 2006; Accepted 28 December 2006

Published online 6 February 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21156.

microinvertebrates harbored by the sponges (Dauby and Jeuniaux, '86). To date the occurrence of chitin in sponges was believed to be limited to a modern group of sponges (the spongillids) that colonized fresh waters from marine environments. Within these sponges, chitin is a component of not the body of the adult but small (300–1,000 μm) resistance bodies called gemmules (Kunike, '25; Rudall and Kenchington, '73). These are produced by the sponges immediately prior to their seasonal disappearance because of extremely severe winters that freeze continental waters or extremely hot summers that drain ponds and creeks. The envelope of gemmules is secreted in the sponge mesenchyme (mesohyl), encapsulating groups of totipotent cells charged with reserves. It consists of a three-layered, non-cellular collagen coat that may even incorporate siliceous spicules in the intermediate layer (Simpson, '84). Chitin is suggested to occur in the inner layer of the gemmule coat (Simpson, '84).

The presence of chitin in gemmules of the Spongillidae but no other anatomical structure of the phylum Porifera points out chitin to be an autapomorphy for this sponge family rather than the ancestral condition within the metazoan lineage. To further examine such a hypothesis, we investigated the potential occurrence of chitin in the internal proteinaceous (spongin) skeleton that either reinforces the mineral skeleton of many demosponges or completely replaces it. In the latter case, which can be found in three taxonomic orders of demosponges (Dictyoceratida, Dendroceratida, and Verongida), the siliceous skeletal pieces that provide support to the soft sponge bodies have evolutionarily been replaced by a skeleton of proteinaceous fibers. Fibers, which may range in thickness from few to several hundred microns, are constituted by densely packed collagen microfibrils arranged within a preferential orientation, usually in concentric layers. The hierarchical, multilevel organization of collagen microfibrils results in spongin, a protein more resistant to enzymatic degradation than collagen microfibrils themselves. Spongin fibers are known to be resistant to bacterial collagenases, pepsin, trypsin, chymotrypsin, pronase, papain, elastase, lysozyme, cellulase, and amylase (Junqua et al., '74).

Fiber chemistry is best known for members of the order Dictyoceratida, with X-ray diffractions and infrared spectra yielding profiles virtually identical to those of other collagens (Garrone, '69). They typically have high content of carbohydrates,

such as galactosyl-hydroxylysine and glucosylgalactosyl-hydroxylysine (Junqua et al., '74). Glucose, galactose, xylose, mannose, and arabinose are also known to occur in fibers (Simpson, '84). However, chitin, a polymer of the aminosugar *N*-acetylglucosamine, often associated with proteinaceous skeletons in several invertebrate groups, has never been found in spongin fibers. The complete absence of chitin from spongin fibers is surprising to some extent, since incorporation of chitin to proteinaceous skeletons of sponges appears to be selectively favored because the resulting material, though somewhat less flexible, becomes more resistant to both pressure and chemicals. Indeed, it is long known that spongin fibers are completely dissolved in 5–20% KOH solutions, while chitin is not (Kunike, '25). Preliminary observations that spongin fibers of some verongid sponges were less flexible and more resistant to dissolution than the well-studied dendroceratid fibers (i.e., bath sponges) prompting us to examine the spongin skeleton of two verongid sponges for the presence of chitin.

MATERIALS AND METHODS

Sample preparation

We examined the fiber skeleton of two demosponges of the order Verongida, *Aplysina* sp., and *Verongula gigantea*, collected from the Lee Stocking Island outer reef of the Bahamas (Fig. 1a and b). In cleaning and preparing the skeletons, we avoided the use of chemicals. The soft tissues of *Aplysina* sp. were slowly eliminated by bacterial attack, as the result of immersing the sponge in seawater at ambient temperature for approximately 2 months, with occasional seawater replacement. The skeleton of *V. gigantea* was found nearly clean at the seafloor of the coral reef, as a result of natural sponge death and subsequent attack by bacteria and scavengers. Both skeletons were finally rinsed in distilled water several times and dried at ambient temperature. Cleaned skeletons had a brownish color (Fig. 1a and b).

Alkaline extraction of sponge fibers

To elucidate the nature of the fiber components, we demineralized fibers by alkali treatments. Fiber skeletons were washed three times in distilled water, cut into $2 \times 2.5 \text{ cm}^2$ pieces, and placed in a 10 ml plastic vessel containing 8 ml of 2.5 M NaOH solution. The vessel was covered to restrict evaporation and placed under thermo-

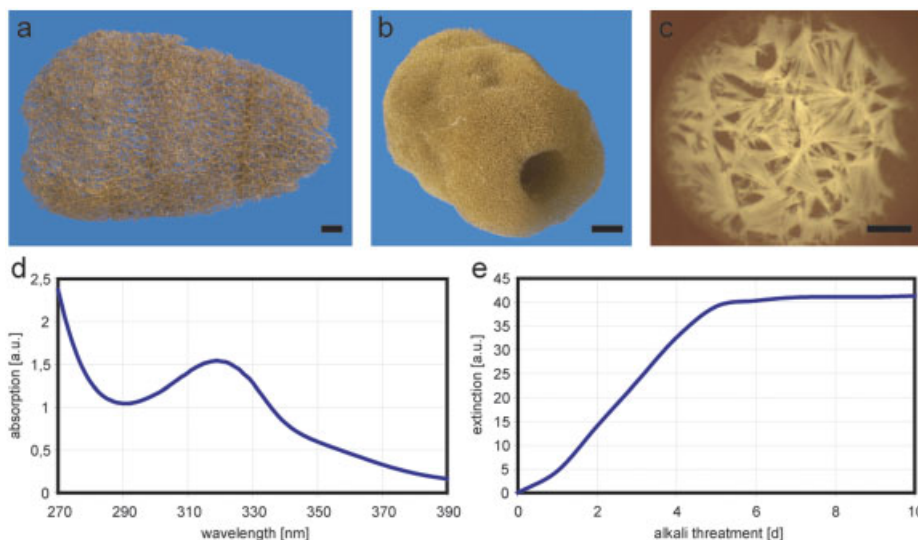


Fig. 1. Macroscopic view of the fiber skeletons of the sponges *Verongula gigantea* (a) and *Aplysina* sp. (b). Absorption spectra of the aeropysinine-like pigment extracted from spongin fibers showing a characteristic peak at 320 nm (d). Measurements at 320 nm show that the fibrous material became pigment-free after the 4th day of its extraction using alkali treatment (e). Calcium carbonate in the form of aragonite (c) was also identified as a component of fibers. Scale bars represent 1 cm.

stated conditions (37°C) with gentle agitation for 7 days. Immersion in NaOH led to an immediate loss of brown pigment from the fibers. Pigmentation loss was further investigated spectrophotometrically using a Perkin Elmer Lambda 10 UV/VIS Spectrometer. The effects of the alkali etching on the fibers were also examined using optical and scanning electron microscopy (SEM) on both pieces of fiber network and fiber's cross-sections. The fibrous colorless material obtained after alkali treatment of the sponge samples was washed with distilled water five times and finally dialyzed against deionized water on Roth (Germany) membranes with a MWCO of 14 kDa. Dialysis was performed for 48 hr at 4°C. The dialyzed material was dried at room temperature and used for staining and analytical investigations. For appropriate comparisons during this and subsequent experiments involved in the process of sponge chitin characterization, we used α -chitin as a control. It was prepared from a commercially available crab shell chitin (Fluka). The material was purified with aqueous 1 M HCl for 2 hr at 25°C and then refluxed in 2 M NaOH for 48 hr at 25°C. The resulting α -chitin was washed in deionized water and centrifugated several times until neutrality was reached. The entire process was repeated twice. β -Chitin was extracted from *Riftia pachyptila* tube-worm and purified at the Institute of Marine Biology FEBRAS (Vladivostok, Russia), following the methodology published elsewhere (Kim et al., '96).

Staining and detection of chitin

To elucidate the particular location of chitin in the fibers, we used dyes that are known to intercalate with polysaccharides. We used Calcofluor White (Fluorescent Brightener M2R, Sigma) which shows enhanced fluorescence when binding to chitin (Hickey et al., 2005). Pieces of untreated clean skeleton and those dialyzed after alkali treatment were placed in 0.1 M Tris-HCl at pH 8.5 for 30 min, then stained using 0.1% Calcofluor White solution for 30 min in darkness, rinsed three times with distilled water, dried at room temperature, and finally observed using Wide Field Fluorescence as well as confocal Laser Scanning Microscopy (LSM) (Zeiss LSM 510 META). For LSM the fluorescence of Calcofluor White was excited by an NIR pulsed laser (770 nm) using two-photon excitation. This corresponds to a single-photon excitation at approximately 380 nm that yields a fluorescence emission at 440 nm.

Immunocytochemistry

The primary antibody is directed against chitin and was developed in rabbits (Buss et al., '96). Fiber portions were first incubated with normal rabbit serum (1:10 diluted) in phosphate buffered saline (PBS) for 4 hr at room temperature to suppress unspecific absorption of the antibodies and then incubated with either the antibody against chitin (1:20 diluted with PBS) overnight

at 4°C or with PBS (control). Both samples were then incubated with an FITC labeled second antibody (goat anti rabbit; Sigma F-1262, diluted 1:50 with PBS) for 4 hr at room temperature.

Chitinase test

Chitinase (EC 3.2.1.14) from the bacterium *Serratia marcescens* was used, one unit of which liberates 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 25°C in a 2 hr assay. Fiber portions of 8.0 mg were incubated with chitinase dissolved in 0.2 M citrate phosphate buffer at pH 4.5 at 25°C for 12 hr or in the buffer as a control. Preparation of colloidal chitin from crab chitin (Sigma) was performed according to Boden et al. ('85). The effectiveness of the enzymatic degradation was monitored using optical microscopy (Zeiss, Axiovert). The Morgan-Elson assay was used to quantify the *N*-acetylglucosamine released after chitinase treatment as described previously (Boden et al., '85).

Fourier transform infrared (FTIR) spectroscopy

IR spectra were recorded with a Perkin Elmer FTIR Spectrometer Spectrum 2000, equipped with an AutoImage Microscope using the Fourier transform infrared reflection absorption spectro-

scopy technique. Fourier transform (FT)-Raman spectra were measured using a Bruker RFS 100/s spectrometer and Nd-YAG excitation at 1,064 nm.

X-ray analysis

X-ray diffraction measurements were performed by an STOE-STUDIP-MP diffractometer with Ge-monochromator at $\text{Cu}_{\text{K}\alpha 1}$ wave length.

Fluorescence and confocal laser scanning microscopy

For both methods an upright light microscope Axioscope 2 FS mot was used. For LSM it was equipped with a Zeiss LSM 510 META scanning head. The fluorescence was excited either by a mercury arc lamp HBO50 or, in the case of LSM, by Ar^+ ion- (488 nm), He/Ne- (546 nm) and Titanium/Sapphire-NIR (770 nm) lasers. The spectra were recorded by the META spectrograph inside the scanning head.

SEM analysis

The samples were fixed in a sample holder and covered with a gold layer for 1 min using an Edwards S150B sputter coater. The samples were then placed in an ESEM XL 30 Philips SEM.

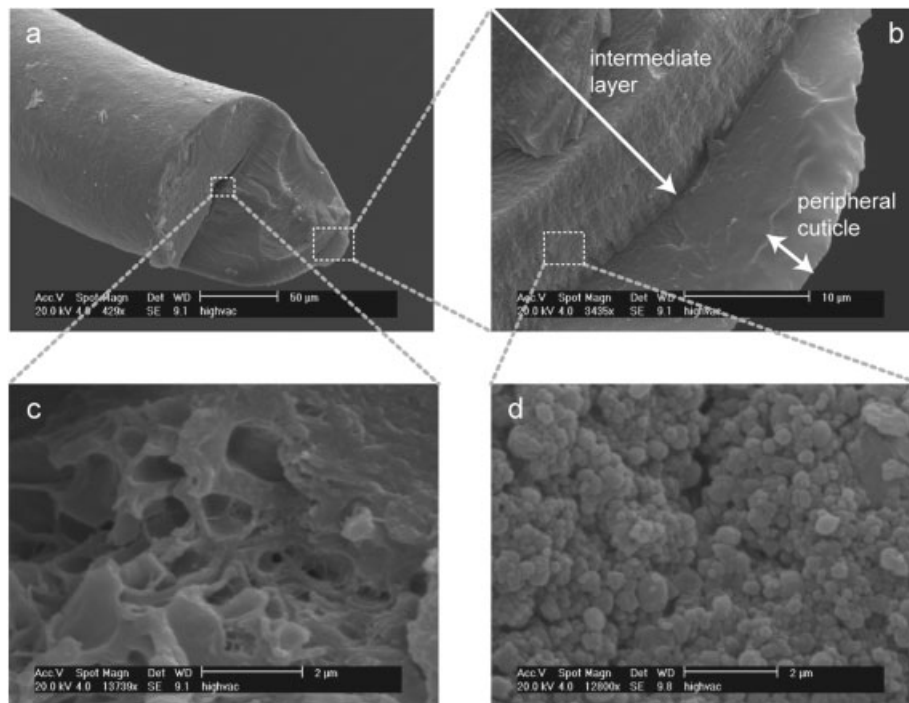


Fig. 2. General SEM view of an *Aplysina* sp. fiber (a) and details of the 5–6 µm thick peripheral cuticle (b), the central 10–15 µm thick porous pith (c) and the crystalline-like 60–70 µm thick intermediate layer (d).

Light microscopy

All material was observed under an MZ12 Leica stereomicroscope using transmitted light.

RESULTS AND DISCUSSION

Loss of pigmentation of the brownish fibers started as soon as they were immersed in NaOH solutions. The pigment showed a characteristic

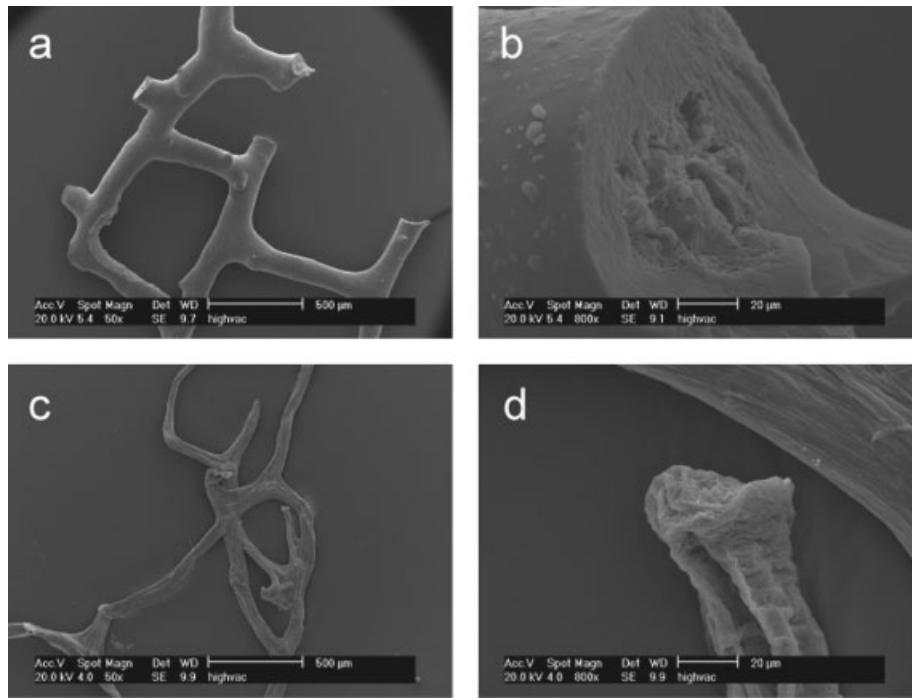


Fig. 3. SEM pictures (a and b) of cleaned, untreated fibers of *Verongula gigantea* compared to equivalent images of NaOH-treated fibers (c and d). Scale bars represent 200 µm.

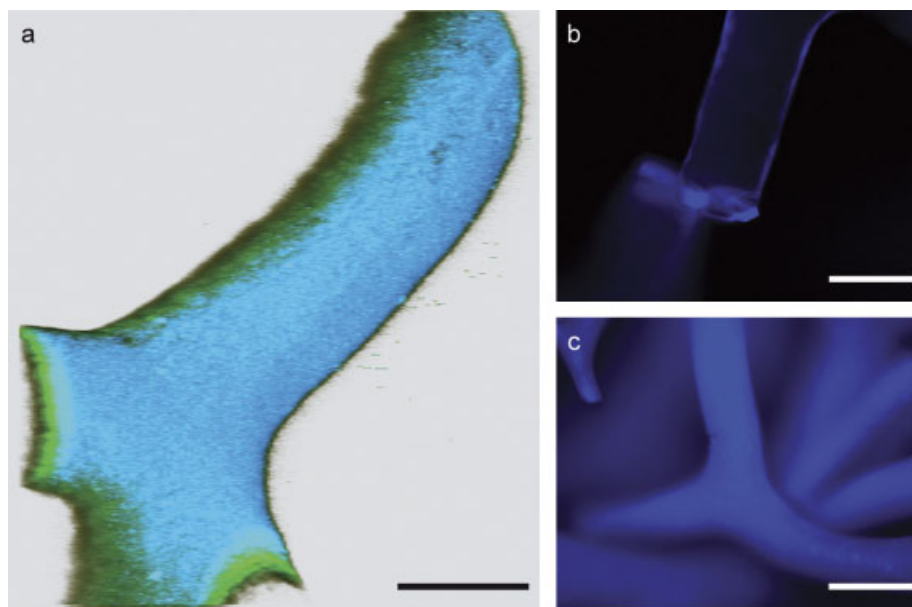


Fig. 4. Calcofluor White staining of the fiber cuticle of *Verongula gigantea* indicating the presence of chitin. (a) 3D reconstruction of a confocal LSM image stack (70 single images) showing calcofluor white fluorescence (light blue) in the outermost region of the fibre as well as natural autofluorescence (green) of the fibre. (b) wide-field fluorescence images revealing marked difference between unstained and (c) stained alkali-insoluble sponge fibers. Scale bars represent 200 µm.

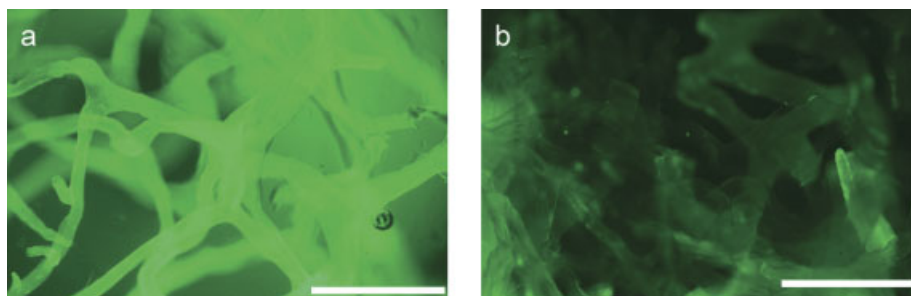


Fig. 6. Immunocytochemical demonstration of chitin showing intense labeling of demineralized and purified *V. gigantea* fiber material (a) vs. no labeling in the control (b). Scale bars represent 1 mm.

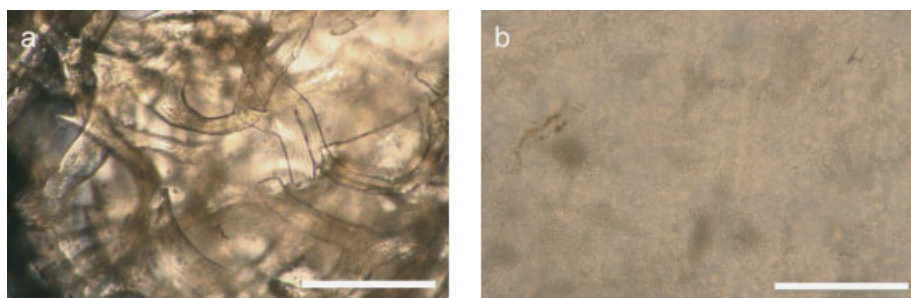


Fig. 7. Comparative view of demineralized and purified fibers of *Verongula gigantea* before (a) and after being treated with chitinase (b). Scale bars represent 1 mm.

infrared spectroscopy as well as X-ray diffraction data, two types of chitin, namely α -chitin and β -chitin, are identifiable (Blackwell, '73). The former is the most common in nature, occurring, for instance, in fungi and in the cuticle of arthropods (Saito et al., '95, 2000). β -chitins appear to be present in a smaller variety of organisms, occurring in squid pens, pogonophoran and vestimentiferan tubes, in setae of some polychaetes, and frustule spines of some diatoms (Saito et al., '95). These polymorphs have a much more open structure (i.e., parallel chain alignment) than α -chitin (anti-parallel chain alignment) (Lamarque et al., 2004). Upon dissolution or extensive intracrystalline swelling, β -chitin converts itself invariably into α -chitin, but once the α -conformation has been reached the change cannot be reverted (Saito et al., '97).

FTIR and Raman spectra of the demineralized fiber material (Table 1, Fig. 5a and b) were compared with spectra for α - and β -chitin spectra, also with those in the literature (Galat and Popowicz, '78; Brugnerotto et al., 2001; Gardenas et al., 2004; Beaney et al., 2005; De Gussem et al., 2005; Wanjuna et al., 2005; Yamaguchi et al., 2005). In the spectrum of *V. gigantea*, the amide bands ascribed to the CONH group vibrational modes appeared at 1,661 and 1,623 cm^{-1} (amide I),

1,560 cm^{-1} (amide II), and between 1,315 and 1,200 cm^{-1} (amide III). Four strong bands ascribed to the C–O–C and C–O stretching modes appeared at 1,157, 1,117, 1,084, and 1,039 cm^{-1} . The vibrational absorption band at 1,382 cm^{-1} was interpreted as the rocking of the methyl group (Brugnerotto et al., 2001). These bands were identical to the reference spectrum of α -chitin, which clearly differentiates from the spectrum of β -chitin because: (1) The amide I band at 1,623 cm^{-1} is more intense in the former; (2) The second maximum of the amide II band at 1,519 cm^{-1} of β -chitin and does not appear in α -chitin; (3) The maximum of the δ CH_2 vibrations is shifted in β -chitin from 1,419 to 1,455 cm^{-1} ; (4) The shape of the amide III region is different in α -chitin and β -chitin; (5) The intense γ CH_x vibration at 962 cm^{-1} appears in α -chitin only.

The FT-Raman spectra for α -chitin and *V. gigantea* fiber material (Fig. 5b) were nearly identical, also in agreement with published reports on chitin identification using Raman spectroscopy (Galat and Popowicz, '78; De Gussem et al., 2005). The FT-Raman spectrum of β -chitin are relatively similar to that of α -chitin, also to that of the sponge, but again significant differences can be found: (1) The maximum of the amide I band is shifted from 1,670 cm^{-1} in β -chitin

TABLE 1. Wave numbers from FTIR- and FT-Raman spectra of β -chitin, α -chitin, and *Verongula gigantea* fibers

β -chitin		α -chitin		<i>V.gigantea</i> demineralized skeleton material		Vibrational assignment
IR	Raman	IR	Raman	IR	Raman	
	330.9		323.2		323.1	δ CCC ring deformation
	362.1		367.3		367.3	δ CCC ring deformation
	388.0		397.5		402.9	δ CCC ring deformation
	413.1		—		—	δ CCC ring deformation
	456.5		454.8		454.8	δ CCC ring deformation
	503.0		500.8		500.5	C–C backbone
	643.7		—		—	C–O, C–C, C–H bending
	828.3		—		—	C–O, C–C, C–H bending
	853.8		—		—	C–O, C–C, C–H bending
891.0	891.9	895.7	895.0	896.9	896.5	γ CH _x
	924.5		—		—	γ CH _x
—	958.4	951.9	953.9	950.6	953.8	γ CH _x
976.2		975.9		975.7		γ CH _x
	1004.3		—		—	C–O–C, C–O stretching
1,027.1	1027.0	1039.1	1030.1	1039.4	1034.1	C–O–C, C–O stretching
	1047.7		—		—	C–O–C, C–O stretching
1,077.3	1063.0	1084.4	1060.8	1084.4	1060.8	C–O–C, C–O stretching
1,116.7	1107.6	1116.7	1112.0	1116.7	1112.0	C–O–C, C–O stretching
	1135.6		—		—	C–O–C, C–O stretching
1,152.5	1152.3	1157.3	1150.2	1157.3	1150.2	C–O–C, C–O stretching
	1174.9		—		—	C–O–C, C–O stretching
1,202.7	1203.8	1203.8	1203.8	1203.8	1203.8	Amide III
1,240.9	1245.6	1237.3	—	1237.3	—	Amide III
1,260.5	1269.4	1261.1	1265.5	1262.3	1265.4	Amide III
1,314.5	1312.5	1314.8	—	1314.9	—	Amide III
	1331.5		1327.6		1327.5	Amide III
	1360.8		—		—	Amide III
1,375.8	1382.5	1380.6	1375.9	1381.8	1375.8	Rocking CH ₂
	1421.4	1418.6	1413.6	1418.5	1415.2	δ CH ₂
1,454.6	1448.5	—	1450.9	—	1452.5	δ CH ₂
1,519.3		—	—	—	—	Amide II
1,559.7	1555.5	1560.1	1560.0	1560.0	1560.0	Amide II
1,623.4		1623.0		1623.0		Amide I
1,667.5	1670.4	1661.5	1658.0	1661.2	1658.0	Amide I
2,878.4		2879.3		2879.5		CH _x stretching
2,932.6		2932.8		2932.6		CH _x stretching
2,963.4		2962.9		2962.8		CH _x stretching

to 1,658 cm⁻¹ in α -chitin (2) The maximum of the amide II band is shifted from 1,555 cm⁻¹ in β -chitin to 1,560 cm⁻¹ in α -chitin; (3) The rocking CH₂ vibrations are shifted from 1,382 cm⁻¹ in β -chitin to 1,376 cm⁻¹ in α -chitin (4) Additional bands appear at 1,361, 1,175, 1,136, 1,048, 1,004, 924, 854, 828, and 644 cm⁻¹ in the spectrum of β -chitin. Similar results (data not shown) were obtained for *Aplysina* sp. fibers.

The X-ray diffractograms of both *V. gigantea* and *Aplysiona* sp. (Fig. 5c) fibers were largely similar to diffractograms of α -chitin described in the literature (Feng et al., 2004; Lima and Airoidi, 2004; Muzzarelli et al., 2004). Nine peaks were

observed, three of which can be interpreted as an ideal crystalline phase (d -values at 5.5, 4.2, 3.8 Å). Six peaks appear broader, which is likely caused by polycrystalline domains or disturbed crystal structure, such as either a paracrystalline phase or the semicrystalline ordering known from synthetic polymers. The broad peaks show d -values at 10.8, 7.0, 4.6, 3.4, 2.5, and 2.3 Å. Some of the peaks were identified and indexed, as follows: (020) corresponding to 10.8/10.2 Å, (110) and (040) to 4.6 Å, and (101) to 4.2 Å (23, 24). The peaks at 7.0, 5.5, 4.2, and 3.8 Å possibly correspond to (021), (031), (111), and (130), respectively. The peaks at 3.4, 2.5, and 2.3 Å in the investigated sponge

material could not be indexed. Our measured diffractograms were remarkably similar to that of Lima and Airolidi (2004). Nevertheless, the peak at 3.8 Å was not as broad as that measured by these authors, presumably because of the higher crystallinity of the sponge chitin. A comparison of the diffractograms of *V. gigantea* and *Aplysina* sp. reveals a shift of the 10.8 Å peak to higher 2θ values corresponding to 10.2 Å for *Aplysina* sp. Moreover, the peak with the d -value 5.5 Å is absent in the case of *V. gigantea* and finding not reported by Lima and Airolidi (2004). The peaks with d -values at 10.8 and 4.6 Å were more intense for *Aplysina* sp. than for *V. gigantea*, likely because of decreased crystallinity of the chitin of the former relative to the latter one.

All together, our findings indicate a remarkable similarity in structure between the chitin found in the fibers of the studied sponges and that the α -chitin reported from other invertebrates. How the chitin of the spongin fibers of these marine sponges relates with the chitin reported from the gemmule wall of fresh-water sponges remain to be addressed. More importantly, our results suggest that occurrence of a chitin-production system is an ancestral condition in Metazoa. To date, no clear mechanism for the biogenesis of α -chitin microfibrils has been established despite the abundance of this allomorph in nature (Saito et al., '97). Because fiber skeletons of marine sponges have recently been used as biomimetic scaffolds for human osteoprogenitor cell attachment, growth and differentiation, showing specific elastomeric and bioactive properties for potential applications in biomedicine and material sciences (Green et al., 2003), we are urged to attain better understanding of the synthesis, chemical composition, and structure of these chitin-bearing fibers.

ACKNOWLEDGMENTS

We thank O. Trommer, T. Douglas, G. Richter, H. Meissner, H. Zimmermann, A. Mensch for their technical assistance, and A. Ereskovsky and K. Tabachnik for discussion.

LITERATURE CITED

- Beaney P, Lizardi-Mendoza J, Healy M. 2005. Comparison of chitins produced by chemical and bioprocessing methods. *J Chem Technol Biotechnol* 80:145–150.
- Blackwell J. 1973. The polysaccharides. In: Walton AG, Blackwell J, editors. New York, London: Academic Press.
- Boden N, Sommer U, Spindler K-D. 1985. Demonstration and characterization of chitinases in the *Drosophila* Kc cell line. *Insect Biochem* 15:19–23.
- Brugnerotto J, Lizardi J, Goycoolea FM, Argüelles-Monal W, Desbrieres J, Rinaudo M. 2001. An infrared investigation in relation with chitin and chitosan characterization. *Polymer* 42:3569–3580.
- Buss U, Varum KM, Peter MG, Spindler-Barth M. 1996. ELISA for quantitation of chitin, chitosan and related compounds. *Adv Chitin Sci I*:254–261.
- Dauby P, Jeuniaux C. 1986. Origine exogene de la chitine d'cel'e chez les Spongiares. *Cah Biol Mar* 28:121–129.
- De Gussem K, Vandenebeele P, Verbeke A, Moens L. 2005. Raman spectroscopic study of *Lactarius* spores (Russulales, Fungi). *Spectrochim Acta A Mol Biomol Spectrosc* 61:2896–2908.
- Ehrlich H, Maldonado M, Hanke T, Meissner H, Born R, Scharnweber D, Worch H. 2003. Spongins: nanostructural investigations and development of biomimetic material model. *VDI Berichte* 1803:287–292.
- Feng F, Liu Y, Hu K. 2004. Influence of alkali-freezing treatment on the solid state structure of chitin. *Carbohydr Res* 339:2321–2324.
- Galat A, Popowicz J. 1978. Study of the Raman scattering spectra of chitins. *Bull Acad Pol Sci Biol* 26:519–524.
- Gardenas G, Cabrera G, Taboada E, Miranda SP. 2004. Chitin characterization by SEM, FTIR, XRD and 13C cross polarization/mass angle spinning NMR. *J Appl Polym Sci* 93:1876–1885.
- Garrone R. 1969. Collagene, spongine et squelette mineral chez l'éponge *Haliclona rosea* (O.S.). *J Micron* 8:581–598.
- Green D, Howard D, Yang X, Kelly M, Oreffo RO. 2003. Natural marine sponge fiber skeleton: a biomimetic scaffold for human osteoprogenitor cell attachment, growth, and differentiation. *Tissue Eng* 9:1159–1166.
- Herth W, Zugenmaier P. 1977. Ultrastructure of the chitin fibrils of the centric diatom *Cyclotella cryptica*. *J Ultrastruct Res* 61:230–239.
- Hickey PC, Swift SM, Roca MG, Read ND. 2005. Live-cell imaging of filamentous fungi using vital fluorescent dyes. *Method Microbiol* 34:63–87.
- Jeuniaux C. 1963. Chitine et Chitinolyse: un Chapitre de la Biologie Moleculaire. Paris: Masson et Cie.
- Junqua S, Robert L, Garrone R, Pavans de Ceccatty M, Vacelet J. 1974. Biochemical and morphological studies on collagens of horny sponges. *Ircinia* filaments compared to spongines. *Connect Tissue Res* 2:193–203.
- Kim SS, Kim SH, Lee YM. 1996. Preparation, characterization and properties of beta-chitin, chitosan and *N*-acetylated beta-chitin. *J Polym Sci, Part B: Polym Phys* 34:2367–2374.
- Kunike G. 1925. Nachweis und Verbreitung organischer Skelettsubstanzen bei Tieren. *Zeitschr Vergl Physiol* 2:233–253.
- Lamarque G, Viton C, Domard A. 2004. Comparative study of the second and third heterogeneous deacetylations of alpha- and beta-chitins in a multistep process. *Biomacromolecules* 5:1899–1907.
- Lima IS, Airolidi C. 2004. A thermodynamic investigation on chitosan-derived cation interactions. *Thermochem Acta* 421:133–139.
- Muzzarelli C, Francescangeli O, Tosi G, Muzzarelli RAA. 2004. Susceptibility of dibutyl chitin and regenerated chitin fibres to deacetylation and depolymerization by lipases. *Carbohydr Polym* 56:137–146.

- Nguyen TH, Fleet GH, Rogers PL. 1998. Composition of the cell walls of several yeast species. *Appl Microbiol Biotechnol* 50:206–212.
- Peter MG. 2002. Chitin and chitosan in fungi. In: Steinbüchel A, editor. *Biopolymers, Vol. 6: Polysaccharides II*, Weinheim: Wiley-VCH.
- Rudall KM. 1955. *The distribution of collagen and chitin*. Cambridge: Cambridge University Press.
- Rudall KM, Kenchington W. 1973. The chitin system. *Biol Rev* 49:597–610.
- Saito Y, Okano T, Chanzy H, Sugiyama J. 1995. Structural Study of alpha-chitin from the grasping spines of the arrow worm (*Sagitta* spp.). *J Struct Biol* 114:218–228.
- Saito Y, Putaux JL, Okano T, Gail F, Chanzy H. 1997. Structural aspects of the swelling of beta-Chitin in HCl and its conversion into alpha-chitin. *Macromolecules* 30:3867–3873.
- Saito Y, Okano T, Gail F, Chanzy H, Putaux JL. 2000. Structural data on the intra-crystalline swelling of beta-chitin. *Int J Biol Macromol* 28:81–88.
- Simpson TL. 1984. *The cell biology of sponges*. New York: Springer-Verlag.
- Wagner GP. 1994. Evolution and function of the chitin system. In: Schrierwater B, Streit B, Wagner GP, De Salle R, editors. *Basel: Birkpuser Verlag*.
- Wanjuna T, Cunxin W, Donghua C. 2005. Kinetic studies on the pyrolysis of chitin and chitosan. *Polym Degrad Stabil* 87:389–394.
- Willmer P. 1990. *Invertebrate relationships*. Cambridge: Cambridge University Press.
- Yamaguchi Y, Nge TT, Takemura A, Hori N, Ono H. 2005. Characterization of uniaxially aligned chitin film by 2D FT-IR spectroscopy. *Biomacromolecules* 6:1941–1947.