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The demosponge *Pseudoceratina purpurea* as a new source of fibrous chitin

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ABSTRACT

Among marine demosponges (Porifera: Demospongiae), only representatives of the order Verongiida have been recognized to synthetize both biologically active substances as well as scaffolds-like fibrous skeletons made of structural aminopolysaccharide chitin. The unique 3D architecture of such scaffolds open perspectives for their applications in waste treatment, biomimetics and tissue engineering. Here, we focus special attention to the demosponge *Pseudoceratina purpurea* collected in the coastal waters of Singapore. For the first time the detailed description of the isolation of chitin from the skeleton of this sponge and its identification using diverse bioanalytical tools were carried out. Calcofluor white staining, FTIR analysis, electrospray ionization mass spectrometry (ESI-MS), SEM, and fluorescence microscopy as well as a chitinase digestion assay were applied in order to confirm with strong evidence the finding of alpha-chitin in the skeleton of *P. purpurea*. We suggest that the discovery of chitin within representatives of Pseudoceratinidae family is a perspective step in evaluation of these verongiid sponges as novel renewable sources for both chitin and biologically active metabolites, which are of prospective use for marine oriented biomedicine and pharmacology, respectively.

1. Introduction

The presence of chitin in some sponge macerates was previously explained as "contamination" by a variety of micro-invertebrates (such as copepod crustaceans or nematods) harbored by or living in the sponges [1]. The first observation of chitin-based scaffolds as an integral part of skeletal elements of several sponges was reported only in 2007 [2,3]. A system and comprehensive studies among the representatives of four classes of sponges (Hexactinellida; Calcarea; Demospongiae: Homoscleromorpha) continue up to date [4–7]. Chitin-based scaffolds possess the shape and morphology which closely resemble to the shape of the source marine demosponge species. These unique, naturally pre-structured scaffolds can be easy isolated from the sponge skeletons using a stepwise extraction procedure based on the use of 2.5 M NaOH [2]. This treatment doesn't interfere with or chemically decompose chitin. The concentration of 2.5 M is even below the threshold concentration where the transformation of β-chitin into α-chitin starts to take place [8]. Spectroscopy, as well as X-ray diffraction consistently shows that sponge chitin is much closer to α-chitin known from other animals than to β-chitin [4,9].

Marine sponges of Verongiida order can produce unique, chitin-based, highly organized 3D networks composed of cross-linked fibers approximately 40–100 μm in diameter [10]. These fibers consist of

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loosely packed chitin, with rough, deeply fissured surfaces. The tubular chitinous scaffolds isolated from demosponges possess unique fibrillar morphology with numerous chambers, channels, and the ability to swell. The complex network of tubes and chambers acts like a capillary and swelling in these skeletons is the result of capillary action. It was proposed to use it as reservoir for different types of liquids and gel-forming media which in turn could contain biotechnologically useful cells, bacteria, yeast, or electrolyte solutions for subsequent mineral formation or metallization of the nanostructured fibrous surfaces [11,12]. Newly discovered chitinous scaffolds from marine sponges are significant for their potential use for 3D cell cultures and tissue engineering. Moreover, the sponge chitin has many advantages over synthetic polymer solutions, including its natural 3D-structure, reproducibility, biodegradability, biocompatibility as well as its sustainable extraction. This chitin is thermostable up to 400 °C [13] and therefore useful for high temperature sterilization techniques. Experiments with selected chitin-based scaffolds of poriferan origin carried out in the lab of prof. Ehrlich group during the last five years with respect to cultivation of different cell cultures show promising results. For example, it was shown for the first time [5] that freshly isolated chondrocytes attached to the *Aplysina cauliformis* sponge chitin scaffolds and extracellular matrix similar to that found in other cartilage tissue of engineering constructs. Chitin-based scaffolds also support deposition of a proteoglycan-rich extracellular matrix of chondrocytes seeded bioconstructs *in vivo* [14]. It was shown [15] that the scaffolds based on chitinous skeletons derived from marine sponges of Verongiida order promote adhesion, proliferation and differentiation into osteogenic and adipogenic directions of human adipose tissue-derived mesenchymal stem cells, which could provide broad opportunities for design of new biocompatible and functionally active bioengineered structures [16,17]. Thus, the demosponge order Veongiida with its four families [18] (Fig. 1) seems to be rich on species which produce chitinous skeletal scaffolds. Aplysinidae is the largest verongiid family (46 species in three genera: *Aplysina*, *Verongula*, *Aiolochroia*) defined by an anastomosing fiber skeleton with both pith and bark elements. Ianthellidae is the second largest verongiid family (21 species in four genera: *Anomoianthella*, *Hexadella*, *Ianthella*, and *Vansoestia*). This family is distinguished from other verongiid families by the presence of eurypylous choanocyte chambers. Aplysinellidae consists of 17 species in three genera (*Aplysinella*, *Porphyria* and *Suberea*) and distinguishing by precence of a dendritic fiber skeleton with both pith and bark elements [19-21]. The family Pseudoceratinidae is monotypic and represented by the genus *Pseudoceratina* currently including four species in the genus and is easily defined by a dendritic fiber skeleton with only pith elements [19]. Up to today, attempts to extract and identify chitin from any member of the genus *Pseudoceratina* have not been reported. Consequently, here we represent the first analytical study on isolation and identification of chitin from the verongiid demosponge *Pseudoceratina purpurea*, Carter 1885. The discovery of chitin within skeletal fibers of this sponge can suggest that chitin is a characteristic feature of the family Pseudoceratinidae as well.

2. Materials and methods

2.1. Sample collection and preparation

The sample of *Pseudoceratina purpurea* Carter, 1885 was collected by SCUBA from the St. John's Island in Singapore (Coll. Swee-Cheng Lim) on June 15, 2017 from the populations located on the depth of 1 m. After collection samples were pack into Ziplock bags and stored on ice during transport to the laboratories of the Tropical Marine Science Institute, Singapore. The specimens were carefully inspected with respect to the intactness of their skeletons and the presence of algae or invertebrates using a stereomicroscope. After this inspection, the sponge materials were frozen at −20 °C and freeze-dried prior to their transfer to the bioanalytical laboratories in Freiberg (Germany) (Supplementary Fig. 1).

2.2. Isolation of chitin skeleton from P. purpurea

The isolation of chitin-based skeleton from *P. purpurea* (Supplementary Fig. 1) was carried out as previously described. [6,22–23] The methodology can be divided into 4 steps (Supplementary Fig. 2): firstly, the sponge skeleton was placed in deionized water at room temperature for 1 h in order to remove possible water-soluble sediment particles and salts. Then, the samples were treated with 20% acetic acid at room temperature for 12 h in order to remove residual carbonate-based debris (microfragments of crustacean carapaces and mollusc shells) from the skeleton of *P. purpurea*. Afterwards, the samples were washed several times with deionized water until achieving a pH of 6.5. At the next step, these samples were treated with 2.5 M NaOH at 37 °C for 72 h to remove pigments and proteins. The foreign siliceous spicules

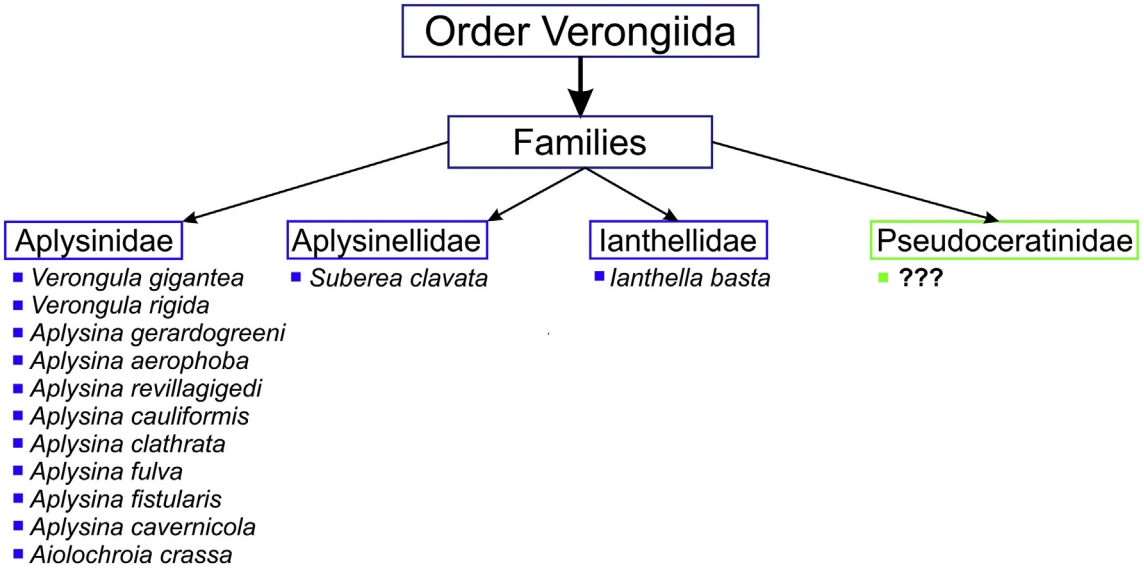

Fig. 1. The order Verongiida comprises four families distinguished mainly on the basis of the structure and composition of their skeletal fibers [4,28]. Up to date, chitin have been isolated and identified only in representatives of three families listed in the schema. No reports or studies about occurrence of chitin within skeletons of sponges belonging to the family Pseudoceratinidae are available in the literature.

and sand microparticles were observed in the samples after 72 h of alkali treatment. Thus thorough, additional desilicification was needed. Consequently, alkali-treated samples were accurately rinsed with deionized water and placed into a plastic vessel containing appropriate amount of 2% hydrofluoric acid (HF) solution. The vessel was covered in order to prevent the evaporation of HF. Desilicification was conducted at room temperature for 12 h. The influence of alkaline and strong acidic treatments on the structure of skeleton of the studied demosponge was investigated using stereo, white light and fluorescence microscopy. Finally, the isolated material was washed several times with deionized water up to a pH level of 6.5. The fibrous translucent scaffolds (Fig. 2) were placed into 250 ml large GLS 80 Duran glass bottles containing deionized water and stored at 4° C for further analyses.

2.3. Light and fluorescent microscopy imaging

Collected sponge samples and isolated chitinous scaffolds have been observed using BZ-9000 microscope (Keycence) in light as well as in fluorescent microscopy modus.

2.4. Calcofluor white (CFW) staining

In order to evaluate the location of chitin in the demineralized skeleton of *P. purpurea* demosponge, Calcofluor White (Fluorescent Brightener M2R, Sigma) was used, because it exhibits enhanced fluorescence when linked to chitin [24,25].

2.5. Scanning electron microscopy

The surface morphology and microstructure of isolated chitinous scaffolds as well as untreated samples of *P. purpurea* were investigated on the basis of SEM images using ESEM XL 30 Philips scanning electron microscope. Before analysis, samples were covered with a carbon layer for 1 min using Edwards S150B sputter coater.

2.6. Fourier-transformation infrared spectroscopy

Transmission spectra of chitinous scaffolds were made using a Nicolet 210c FTIR spectrometer. The samples were analysed using ATR system.

2.7. Chitinase digestion and test

Chitinase from the fungus *Trichoderma viride* (EC 3.2.1.14, No. C-8241, Sigma–Aldrich, Germany) was used. For details please see Supplementary Information.

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

The Morgan–Elson assay was used in order to quantify the *N*-acetyl-D-glucosamine released after chitinase treatment, as described previously [2,23,26]. For details see Supplementary Information.

3. Results and discussion

It is recognized that demosponges of the order Verongiida together with the orders Chondrillida and Chondrosiida belong to the subclass Verongimorpha is a clade of aspiculate demosponges [19]. Consequently, our observations of some spicules located within NaOH-treated skeletal fragments of *P. purpurea* (Supplementary Fig. 3) confirm the suggestion about the allochtonic origin of these structures.

The use of foreign usually sandy particles (siliceous or calcareous) taken from the surrounding environment as foreign material for skeletal carcass is an innovative solution successfully developed independently more than once in different groups of animals (*e.g.* testate amoeboids, foraminifera and sponges). The benefit of such an evolutionary novelty is apparently due to the simplicity of use of constructive material for making of which not need to waste own material and energy. Meanwhile among multicellular organisms the greatest success in applying foreign rather than synthesized material in construction of internal skeletal elements was achieved by diverse sponges [19,27–29]. It is shown that the foreign particles are captured by sponge and in some cases transferred by means of a special transport mechanism into which special carrying cells are involved [30]. However, our bioanalytical data represented below showed the chitinous nature of the *P. purpurea* skeleton. Consequently, our finding of foreign particles attaching to fibrous chitin of *P. purpurea* demosponge pointed to the existence of more complex mechanism for incorporation of foreign particles into the skeleton of sponges. It is noteworthy that we never observed foreign materials in other verongiid sponges with chitin-based skeletons. The skeletons of those species contain silica- and calcium carbonate-based biominerals produced by investigated sponges [7].

SEM microphotographs of the skeletal fibers of *P. purpurea* prior and after HF-based treatment are shown on Fig. 3. Only HF-based treatment leaded to disappearance of the foreign spicules and sandy

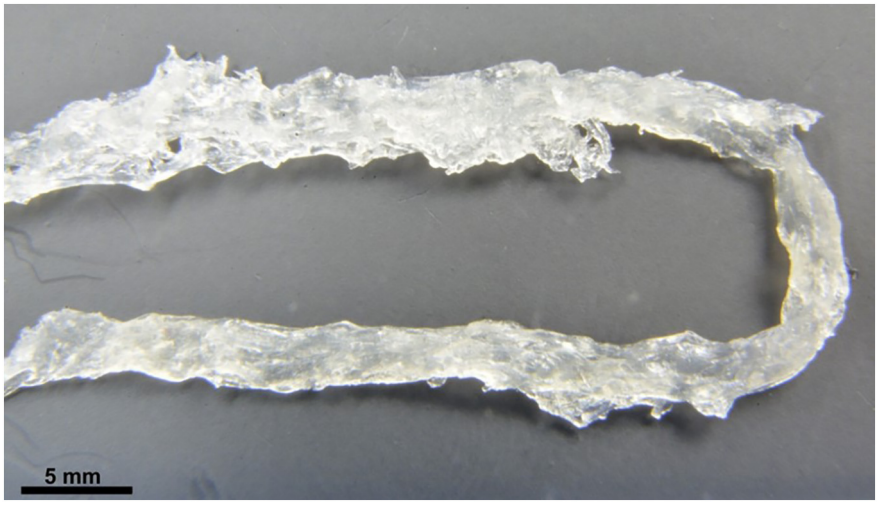

Fig. 2. Completely pigment-free and demineralized scaffold isolated from *P. purpurea* demosponge still resembles the morphology and the shape of the original collected and freeze-dried sponge (see Supplementary Figure1).

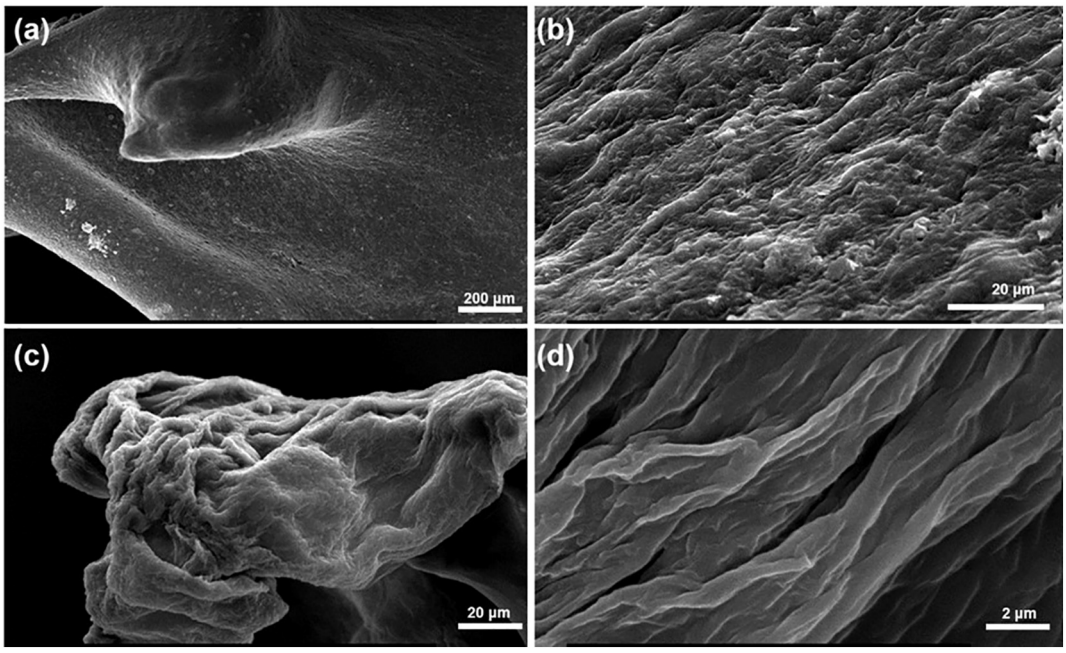

Fig. 3. SEM imagery of the *P. purpurea* skeletal fibers prior (a, b) and after HF-based treatment (c, d).

microparticles as well as the isolation of pure organic microfibers (Fig. 3c, d). The same phenomenon can be observed using white light and fluorescence microscopy (Fig. 4a, b). The nanostructural organization of these microfibers is represented in the Supplementary Fig. 4.

The Calcofluor white staining (CFW) [31] was the first step for the preliminary identification of chitin within isolated and demineralized skeletons. CFW is generally used as a fluorescent dye for staining of β-(1 → 3)- and β-(1 → 4)-linked polysaccharides [32,33]. As a result, on binding to polysaccharides such as chitin, this fluorescent brightener emits a bright blue light under UV excitation. Fluorescence microscopy of the fibrous scaffolds isolated from *P. purpurea* after CFW staining display very strong fluorescence even under light exposure time as short as

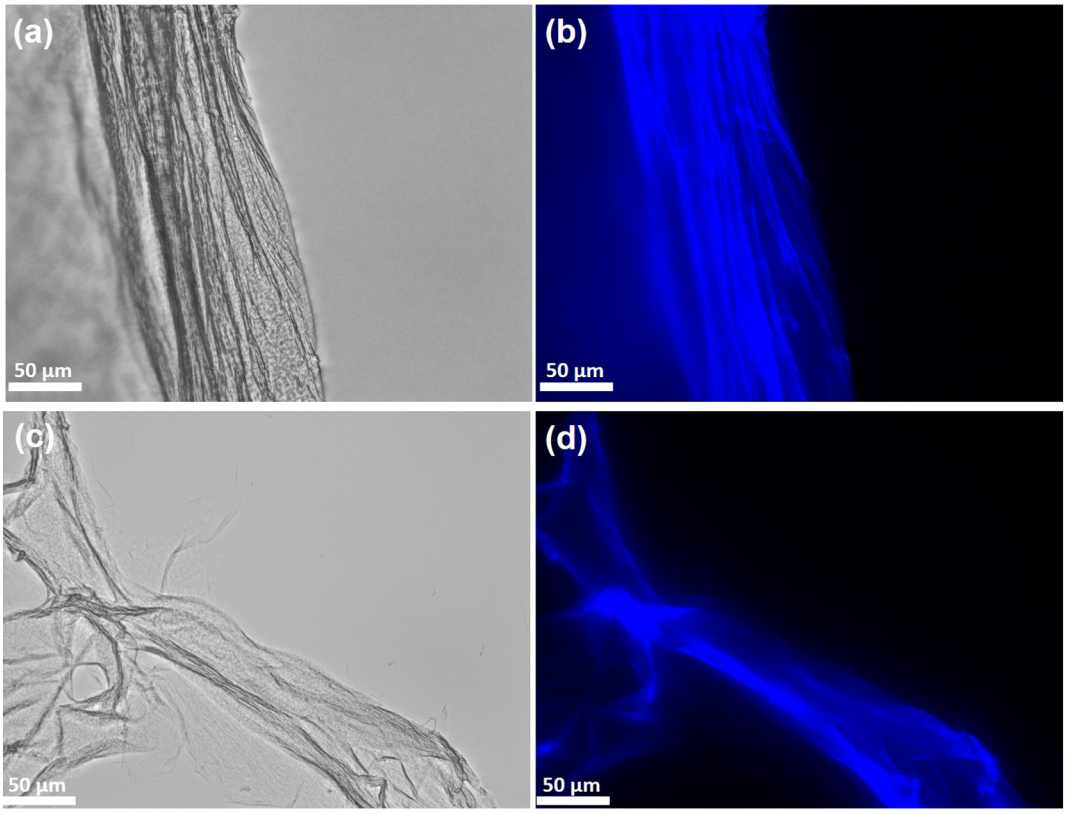

Fig. 4. Purified skeletal fibers of *P. purpurea* (a,c) show typical chitin autofluorescence (light exposure time 1/45) (b) and very intensive fluorescence (light exposure time 1/3700) after Calcofluor white staining for chitin (d).

1/3700 s (Fig. 4d). Similar results were previously reported for chitin isolated from fossilized remnants [31,32] as well as recent species of marine [32,9] and freshwater sponges [23].

To confirm the presence of chitin in the isolated scaffolds (Figs. 3d and 4a), more sensitive analytical techniques were applied. FTIR spectroscopy is one of the powerful tools in the structural analysis of polysaccharides including chitin. Vibrational spectra are also sensitive to intramolecular and intermolecular interactions as well as to the geometry of molecules. Recently, this method was successfully applied for identification of three known isomorphs of chitin (α, β and γ) [34]. FTIR spectra registered for the fibrous material isolated from *P. purpurea*, as well for α-chitin standard are presented in Fig. 5. The region of the amidic moiety, between 1700 and 1500 cm^{-1}, yields different signatures for chitin polymorphs. In this region the spectra of the samples studied by us showed strong adsorption band associated with the stretching vibrations of C═O group characteristic of the amide I band. The amide I band registered for isolated sample showed tween peak (at 1644 cm^{-1} and 1633 cm^{-1}), and it corresponds with spectrum registered for α-chitin polymorph [35,36]. It is worth to note, that in case of β-chitin, reported by [36,37], amide I band is observed in form of single band at 1656 cm^{-1}. Splitting of the amide I is characteristic feature for α-chitin and originates from two types of C═O groups within the biopolymer chain [38]. One is a result of the intermolecular hydrogen bonds between C═O and H—N groups and second results from specific intramolecular hydrogen bond of carbonyl group with the hydroxymethyl group of the next chitin residue of the same chain [39]. Additional indicative feature is the presence of the intense band at 949 cm^{-1} in spectrum registered for isolated sample. This band is assigned to CH_3 wagging along chain and is well visible in α-chitin spectrum at 952 cm^{-1}. Accordingly, to references [36,37] this band is not observed in spectra of β-chitin. Moreover, the α-chitin indicative band assigned to a ß-glycosidic bond is observed at a wavenumber 898 cm^{-1} in FTIR spectra of the scaffolds isolated from *P. purpurea* (Fig. 5). Detailed analysis of the bands indicates that measured spectra of isolated chitinous structures strongly correspond with those registered for the α-chitin reference.

Raman spectra registered for α-chitin; β-match and isolated sample is presented in Supplementary Fig. 5. The obvious differences between α and β isomorphs are seen in amide I and amide III bands. Firstly, the occurrence of two non-equivalent populations of C═O groups (amide I band) is deafly indicated in α-chitin standard and in isolated sample. In contrast, registered Raman band associated with amide I vibration of β-chitin is broad and weak. Secondly, contrary to broad complex amide III band in β-chitin, the stretching vibrations of C—N and bending vibrations N—H are very well separated in case of α-chitin standard and in isolated sample. The involvement of the primary hydroxyl group in two kinds of hydrogen bonding network accounts for the Raman vibration modes of α- and β-polymorphs both in the CH stretching (3000–2600 cm^{-1}) and in the conformation sensitive region (1450–1420 cm^{-1}) [34,38]. Comparative analysis all regions mentioned above confirms that isolated sample is α isomorph.

The high resolution transmission electron micrographs taken for isolated chitin nanofibers are shown in Supplementary Fig. 6. By the fast Fourier transformation of the high-resolution micrographs the spacings of 4.09, 3.45 and 3.25 Å are associated with the orthorhombic structure typical for α-chitin [3,7,33,41].

Chitinase digestion test have been traditionally carried out previously in studies on chitin detection in diverse sponges [2–6,22,9,40], This enzyme possesses the ability to degrade chitin into low molecular weight chitin oligomers including *N*-acetylglucosamine (GlcNAc). The activity of chitinase is clearly visible with light microscopy (Supplementary Fig. 7). This test is definitely an additional confirmation of the chitinous nature of skeletal scaffolds isolated from *P. purpurea*.

The Morgan–Elson assay has been previously described in details [2,41] and it was used as the most accurate methods to estimate the GlcNAc released after chitinase treatment. Determination of GlcNAc in

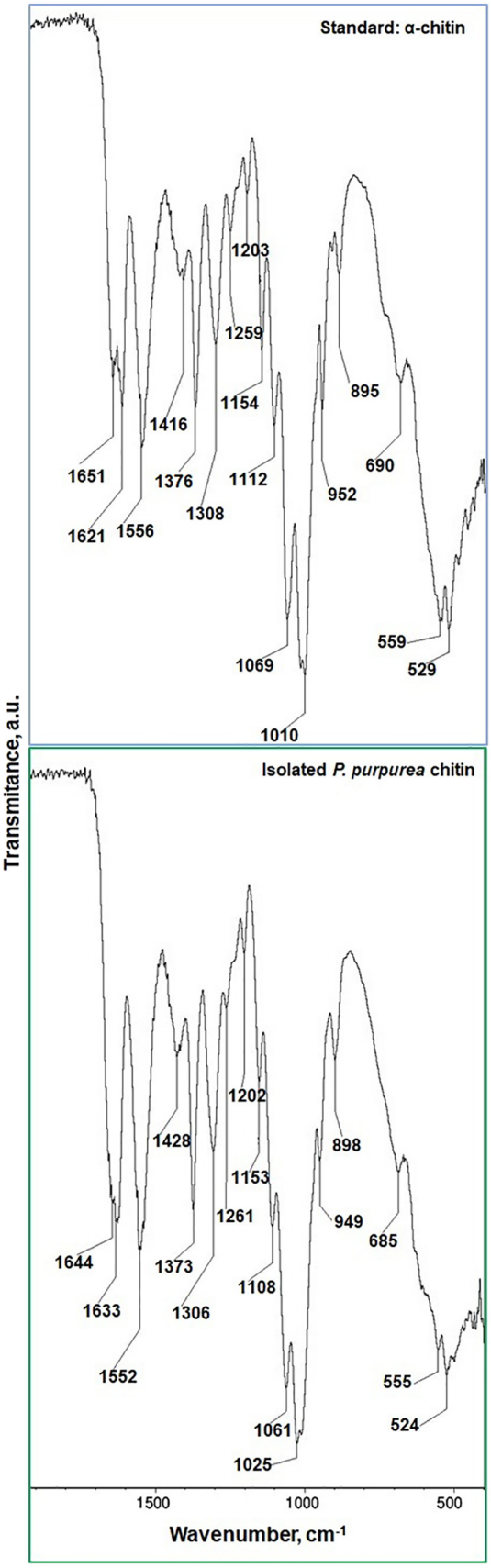

Fig. 5. FT-IR spectra of standard chitin and chitin isolated from the sponge *P. purpurea*.

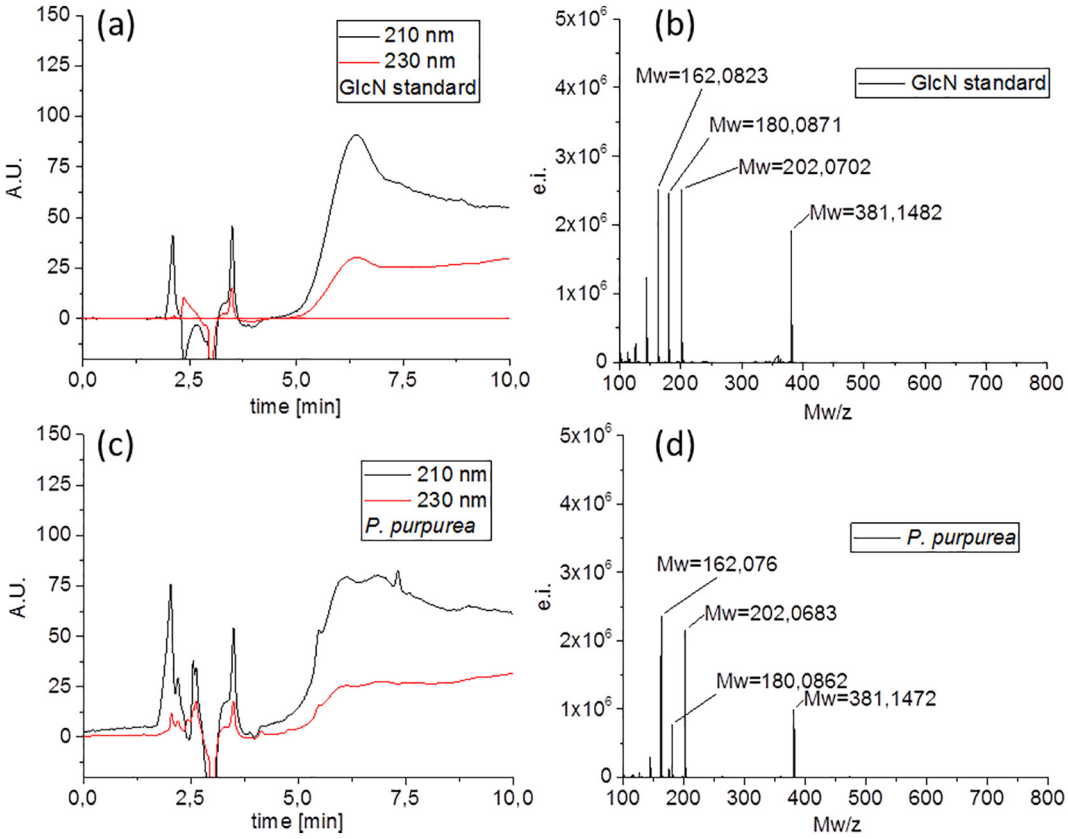

Fig. 6. HPLC and ESI-MS investigations of the chitin isolated from *P. purpurea*. HPLC chromatogram (a) and ESI-MS spectrum (b) of d-glucosamine (dGlcN) standard. HPLC chromatogram (c) and ESI-MS spectrum (d) of acid hydrolysed *P. purpurea* chitin sample (0.1% TFA, H2O/AcN linear gradient).

chitin-based scaffolds of *P. purpurea* showed 950 ± 1.5 μg of *N*-acetyl-glucosamine per mg of chitinous material of this demosponge. These results are similar to that reported for chitin isolated from other verongiid demosponges [4,5,32].

D-glucosamine (GlcN) is the main product of chitin acid hydrolysis which can be separated by HPLC and visualized by ESI-MS. This method was successfully used for identification of chitin in fossil remains [6,33,41] as well as in complex organisms, but also can be used to demonstrate the purity of chitin sample. GlcN eluted in HPLC, which was set at 210 and 230 nm, as a single peak at 2.2 min (Fig. 6a). Because of GlcN high hydrophilicity, its elution time was only slightly longer than the zero volume of the column which was measured as 1.5 min. The 230 nm was chosen for the HPLC monitoring because D-glucosamine dose not deposit any absorption at this wavelength while many possible impurities such as sulfides, terpenes or any aromatic compounds have a significant absorption in this region. The HPLC chromatogram of the hydrolyzed *P. purpurea* chitin sample showed similar shape to the standard with a strong peak at 2.2 min (Fig. 6c). As in the GlcN standard, the sample does not show any strong absorption at 230 nm indicating its high purity which was also confirmed by ESI-MS spectroscopy. ESI-MS of d-glucosamine standard typically shows four main signals with m/z = 162.08, 180.08, 202.07 and 381.14 (Fig. 6b). The signal with m/z = 180.08 corresponds to the molecular ions [M + H+] of a species with a molecular weight of 179.07 which is GlcN (calculated: 179.1) while the signal at 202.07 correspond to the same ion paired with Na +. The signal at m/z = 162.06 corresponds to a fragmented ion [M − H_2O + H+] of GlcN without one of water molecule (calculated: 162.1) and the signal at m/z = 381.14 corresponds to [2 M + Na+] specie which is sodium-bound GlcN non covalent dimmer. ESI-MS of *P. purpurea* sample (Fig. 6c) was nearly identical to the spectrum of GlcN standard. Together, HPLC chromatogram and ESI-MS clearly indicates the purity of the chitin in the studied sample.

Sponges of the genus *Pseudoceratina* are, probably, among the richest sources of secondary metabolites with diverse chemical skeletons and pharmacological activities within the order Verongiida. Representative examples of these secondary metabolites include spermatinamine [42–44], purealidins [45], aplysamine [46,47], ianthesine, aerothionin [48], methyl (2,4-dibromo-3,6-dihydroxyphenyl)acetate [49], ceratinadins [50], psammaplysin [51], ad (1′R,5′*S*,6′*S*)-2-(3′,5′-dibromo-1′,6′-dihydroxy-4′-oxocyclohex-2′-enyl) acetonitrile [52], have been isolated from diverse unidentified species of *Pseudoceratina*. The marine sponges *P. arabica* and *P. verrucosa* have been described as rich producers of different secondary metabolites including phenolic halogenated compounds, moloka'iamine derivatives, psammaplysins, ceratinines [53–55], moloka'iakitamide, aplysterol [53,56], and aplysamine 7, pseudoceratinamide A and B [57,58], respectively. The next table shows representative examples of the secondary metabolites obtained from *P. purpurea* world-wide with reported biological activities (see Table 1). Till now, diverse secondary metabolites of sponge of the genus *Pseudoceratina* listed above have been isolated using traditional organic solvent-based extraction approaches. There are no reports on isolation methods for these metabolites which are based on treatment with alkaline solutions as well as about structural stability of such biomacromolecules at alkaline pH levels. However, experiments with other bromotyrosine- and chitin-producing demosponges representing the order Verongiida showed that bromotyrosines and chitin-based scaffolds could be isolated from the sponge skeletons using a stepwise extraction procedure mainly based on the use of NaOH [68]. Recently, patented method for isolation of both bromotyrosines and chitinous skeletal frameworks from selected sponges, without disruption of the skeletons

Table 1
Biologically active compounds isolated from *P. purpurea* demosponge.

Compounds	Reported biological activity	Ref
Purealdins N, P and Q	Cytotoxic effect on the tumour cell lines	[59]
Purealidins J, K, P and Q	Moderate inhibitory activity against epidermal growth factor receptor kinase	
Ceratinarnides A and B, psammaplysins A and E, ceratinamine, moloka'iamine, pseudoceratidine	Inhibitors of larval settlement and metamorphosis of the barnacle *Balanus amphitrite*	[60]
Psammaplysins E, ceratinamine, moloka'iamine, pseudoceratidine, 4,5-dibromopyrrole-2-carbamide	Inductors of larval metamorphosis of the ascidian *Halocynthia roretzi*	
Pseudoceratidine with two 4.5 dibromopyrrole-2-carbamyl units	Inhibitors of larval settlement and metamorphosis of the barnacle *Balanus amphitrite*	[61]
Ceratinamine	Antifouling activity against *Balanus amphitrite* cyprids and cytotoxicity against P388 murine leukaemia cells	
Tokaradines (bromtyrosines)	Lethal effect on the crab *H. sanguineus*	[62]
Psammaplins A	Histone deacetylase inhibitors and	[63]
Bisaprasin	DNA methyltransferase inhibitors	
Psammaplins F	Histone deacetylase inhibitors	
Psammaplins G	DNA methyltransferase inhibitors	
Aplysamine-2	Moderate *in vivo* anticancer activity	[64]
Ceratamines A and B	Antimiotic effect on cancer cells	[65]
Purpurealidin B	*In vitro* antimicrobial activities against	[66]
16-debromoaplysamine-4	*E. coli, S. aureus, V. cholerae, S. flexineri*	
Purpuramine I	and *S. typhi*	
Purealidin Q	Antimicrobial activity against *S. typhi*	
Aplysamine-2 and aeroplysinin-1	Inhibition of mouse lymphoma cell line L5178Y	[67]
Psammaplin A	HDAC inhibitor	[57]

in the mortar (this being the traditional procedure for extraction of bromotyrosines) has been proposed. [69]

4. Conclusions

There are no doubts about the necessity of the development of novel, more effective technologies for extraction of biologically active compounds together with chitinous scaffolds from *P. purpurea* and related species. Cultivation of *Pseudoceratina* species under marine farming conditions as well as from primmorph- based cultures possesses high potential. The living of *P. purpurea* at low depths (around 1 m) potentially makes the development of the species aquaculture in tropical areas very lucrative and inexpensive. The question about possible technological use of chitinous scaffolds of *P. purpurea* origin as adsorbents, or special thermostable 3D templates for the development of novel, advanced functional composite materials under conditions of extreme biomimetics is still open.

We suggest that the discovery of chitin within other representatives of the genus *Pseudoceratina* would be the next step in the evaluation of the possibility to using these demosponges as a novel renewable source for both biologically active metabolites and chitin having technological and biomedical applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.02.071.

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