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A chromophore-containing agglutinin from *Haliclona manglaris*: Purification and biochemical characterization

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ABSTRACT

A new chromophore-containing agglutinin (*Haliclona manglaris* agglutinin (HMA)) was isolated from the tropical sponge *H. manglaris*. HMA was purified by a combination of hydrophobic interaction chromatography and ion exchange chromatography. Native HMA is a heterotrimer formed by two β-chains (15 kDa) and one α-chain (22 kDa). HMA is a glycoprotein and possesses three intrachain disulfide bonds. Hemagglutinating activity of HMA was stable at neutral pH and temperatures up to 60 °C. HMA was only inhibited by thyroglobulin. Mass spectrometry sequencing and Edman degradation revealed a unique amino acid sequence of about 30%. Moreover, HMA has an organic chromophore of 581 Da, and this characteristic seems to be important to its antioxidant activity. Interestingly, while HMA showed no toxicity against *Artemia* nauplii and was unable to agglutinate bacterial cells, it did show a high capacity to protect β-carotene against oxidation. Thus, our findings suggest the putative involvement of HMA in the protection of the sponge against oxidation.

1. Introduction

Lectins are carbohydrate-binding proteins that are not involved in carbohydrate metabolism, and do not belong to any of the main classes of immunoglobulins [1]. Lectins play several physiological roles, and they are involved in diverse biological processes in animals, such as symbiosis, infection defense, apoptosis and sugar traffic [2–4].

Recently, many lectins have been isolated from marine sources [5–7]. In particular, marine invertebrates have attracted some interest. In these organisms, lectins play a crucial role in innate immune response. They act as opsonins, recognizing foreign substances through binding to their carbohydrate components and triggering phagocytosis of pathogens by scavenger cells [8].

The physiological role of a lectin is typically mediated by the carbohydrate recognition domain (CRD) and by their ability to bind to glycosylated epitopes or other carbohydrate structures. Nevertheless, only a few studies have reported on the ability of lectins to bind to non-glycosylated molecules [9,10]. For instance, the third lectin isolated from the marine sponge *Haliclona caerulea* (H-3) is a blue lectin that recognizes *N*-acetyl-galactosamine. H-3 has color because it interacts with a hydrophobic chromophore of 597 Da (chromophore-597) *via* a domain thus far uncharacterized [11].

The amino acid sequence of H-3 showed high identity with a putative protein encoded in the genome of the sponge *Amphimedon queenslandica*, pAqP, but it showed no significant similarity with any other lectins [11]. Thus, H-3 could not be grouped in any family of animal lectins, *e.g.*, C-type, F-type, L-type, RBL or Galectins.

Porifera represents a basal group of metazoans, and lectins isolated from sponges are among the most ancient protein known. Only a few structural studies have reported on sponge lectins. Nevertheless, some sponge lectins seem to play a physiological role in cellular re-aggregation [12], symbiosis [13] and growth regulation

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[14]. However, up to now, no chromophore-containing lectin has been reported in sponge or other animals. Consequently, the physiological role of chromophore-containing lectins remains unknown.

Therefore, we have searched for lectins similar to H-3, i.e., those having significant amino acid sequence identity and those interacting with chromophores. Here we report a new agglutinin isolated from the tropical sponge *Haliclona manglaris* (HMA). This protein shared properties similar to those of H-3, including a chromophore, two polypeptide chains and glycosylation, but, curiously, HMA and H-3 do not share sequence similarity. We also looked for evidence supporting the physiological roles of these proteins.

2. Material and methods

2.1. Animal collection

Specimens of the marine sponge *H. manglaris* were collected in the intertidal zone of Icaraí Beach, Amontada, Ceará State, Brazil. Fresh sponges were transported on ice to the laboratory and stored at −20 °C until use. The species was identified, and a voucher was deposited (identification number: MNRJ 15936) at the Museu Nacional-UFRJ, Rio de Janeiro, Brazil.

2.2. Isolation of H. manglaris agglutinin

Frozen sponges were cut into small pieces, triturated into powder, and extracted (1:2, w/v) with 20 mM acetate buffer, pH 5.5 (AB). The mixture was strained through nylon tissue and clarified by centrifugation for 20 min at 10,000 × *g* at 4 °C. The supernatant (crude extract) was collected and assayed for hemagglutinating activity (see below) and protein concentration [15].

Ammonium sulfate was added to the crude extract to achieve the final concentration of 500 mM, and the suspension was left for 4 h at room temperature. The precipitated proteins were removed by centrifugation for 20 min at 10,000 × *g* at 4 °C, and the supernatant was loaded on a Phenyl-Sepharose 6B column (1.0 cm × 5.0 cm) equilibrated with 500 mM of ammonium sulfate in AB. The column was washed with the same buffer at a flow rate of 2 mL min^{-1} until the column effluents showed absorbance of less than 0.02 at 280 nm. Two adsorbed fractions (P_1 and P_2) were eluted with 200 mM of ammonium sulfate in AB and ethanol 20%, respectively. The chromatography was monitored at 280 nm, and 3-mL fractions were manually collected and tested for hemagglutinating activity.

The active fraction (P_2) was dialyzed against deionized water, freeze-dried, solubilized in a small volume of 20 mM Tris–HCl buffer, pH 7.6 (TB), and loaded onto a DEAE-Sephacel column (1.0 cm × 3.0 cm) previously equilibrated with TB. The flow rate was adjusted to 1 mL min^{-1}, and the column was washed with TB followed by elution of two adsorbed fractions (D_1 and D_2) with 100 mM and 1 M of NaCl in TB, respectively. The chromatography was monitored at 280 nm and 660 nm, and 1-mL fractions were collected. Fraction D_1 showed hemagglutinating activity and was termed *H. manglaris* agglutinin (HMA).

2.3. Purification of H-3, the blue lectin from Haliclona caerulea

H. caerulea was collected at Pacheco Beach, Caucaia, Ceará State, Brazil. Purified H-3 was obtained using the method previously described by Carneiro et al. [11].

2.4. Hemagglutinating activity and Hemagglutination inhibitory assay

The hemagglutination tests were performed on microtiter plates with V-bottom wells using the two-fold serial dilution method. One hemagglutinating unit (HU) was defined as the amount of lectin able to agglutinate and, hence, precipitate erythrocytes in a suspension after 1 h. Human (A, B and O type) and rabbit erythrocytes were used in native form and treated with papain and trypsin.

A hemagglutination inhibition assay was performed using the standard procedure [16]. The following carbohydrates and glycoproteins were used: D-fructose, D-fucose, D-galactose, D-glucose, D-mannose, methyl-α-D-galactopyranoside, methyl-α-D-glucopyranoside, *N*-Acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-Acetyl-D-mannosamine, D-Sucrose, α-lactose, β-lactose, lactulose, tyroglobulin, ovomucoid and porcine stomach mucin (PSM).

2.5. Molecular mass estimation, sugar content and absorbance spectrum

The molecular mass of HMA under denaturing condition was estimated by SDS-PAGE in 15% gel in the presence and absence of β-mercaptoethanol, followed by staining with Coomassie Brilliant Blue, as described by Laemmli [17]. Homogeneity of the native HMA was evaluated by N-PAGE 12%.

The apparent molecular mass of the native HMA was estimated by size exclusion chromatography on Biosuite™ 250 HR SEC (0.78 × 30 cm, 5 μm particle size) column coupled to an Acquity UPLC™ system (Waters Corp.). The column was equilibrated with 50 mM Tris–HCl, pH 7.2, containing NaCl 500 mM, and calibrated with conalbumin (75 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa).

Neutral carbohydrate content in HMA was evaluated as described by Dubois et al. [18], using lactose as the standard. Glycoproteins in SDS-PAGE were stained with periodic acid-Schiff (PAS), as described by Zacharius et al. [19].

A wavescan was performed using the **Ultrospec™ 2100Pro** UV–Vis **Spectrophotometer** with wavelength ranging from 190 nm to 900 nm to determine maximum absorbance of HMA.

2.6. Effects of pH, temperature and divalent cations on the hemagglutinating activity of HMA

The effects of pH, temperature, EDTA and divalent cations on lectin activity were evaluated as described by Sampaio et al. [16].

2.7. Molecular mass determination

Purified HMA was solubilized with 5% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and submitted to reverse phase chromatography (RPC) coupled to the Acquity system (Waters Corp., Milford, MA, USA). Sephasil Peptide C-8 10/250 column (GE-Healthcare) was equilibrated and washed with ACN 5% containing TFA 0.1% at a flow rate of 1 mL min^{-1}. Retained proteins were recovered by elution through a gradient of 5% to 70% of ACN containing TFA 0.1%. Absorbance of the column eluates was monitored at 216 and 280, and fractions of 0.5 mL were collected.

After separation, HMA chains were vacuum-dried and submitted to mass spectrometry analysis. The average molecular masses of HMA chains were determined using a hybrid Synapt HDMS mass spectrometer (Waters Corp., Milford, MA, USA). HMA chains were solubilized in 50% ACN containing 0.1% formic acid (FA). After centrifugation, protein solutions (10 pmol μL^{-1}) were separately infused at a flow rate of 1 μL min^{-1} into a nanoelectrospray source coupled to a mass spectrometer. The instrument was calibrated with [Glu1]fibrinopeptideB fragments. Mass spectra were acquired by scanning at *m/z* ranging from 500 to 4000 at 2 scan/s. The mass spectrometer was operated in positive mode, using a source temperature of 363 K and capillary voltage at 3.2 kV. Data collection and processing were controlled by Mass Lynx 4.1 software (Waters).

Deconvolution of ESI mass spectra was performed using the MaxEnt 1 algorithm in the Mass Lynx software.

2.8. Quantification of sulfhydryl groups

Free cysteine residues and total cysteine residues in HMA were quantified as described by Carneiro et al. [11].

2.9. N-terminal analysis

Automated Edman degradations were performed with protein sequence Shimadzu model PPSQ-31A (Shimadzu Corp., Japan). PTH-amino acids from the *N*-terminus sequence were separated on a 2.0 × 250 mm ODS column (Wakosil) connected to a model LC-20AT pump. The absorbance was detected at 269 nm with a UV–Vis SPD-20A detector.

2.10. Amino acid sequencing by tandem mass spectrometry (MS/MS)

SDS-PAGE was performed as described above. After staining, HMA spots (α and β chains) were excised, reduced with dithiothreithol (DTT), and carboxyamidomethylated with iodoacetamide (IAA), as described by Shevchenko et al. [20]. Treated spots were subjected to digestion with trypsin (Promega). Digestions were performed in ammonium bicarbonate 50 mM at 1:50 w/w (enzyme/substrate) and maintained at 37 °C for 16 h.

The digestions were stopped with 2 μL of 2% FA. The samples were washed four times with 5% FA in 50% ACN. The supernatants were collected and transferred to fresh tubes, pooled, vacuum-dried, solubilized in 20 μL of 0.1% FA, and centrifuged at 10000 × *g* for 2 min. Two microliters of the peptide solution were loaded onto a C-18 (0.075 × 100 mm) nanocolumn coupled to a nanoAcquity system. The column was equilibrated with 0.1% FA and eluted with a 10% to 85% ACN gradient in 0.1% FA. The eluates were directly infused into a nanoelectrospray source. The mass spectrometer was operated in positive mode with a source temperature of 373 K and a capillary voltage at 3.0 kV. LC–MS/MS was performed according to the data-dependent acquisition (DDA) method. The lock mass used in acquisition was *m/z* 785.84 ion of the [Glu1] fibrinopeptide B. The selected precursor ions were fragmented by collision-induced dissociation (CID) using argon as the collision gas. All of the CID spectra were manually interpreted and searches for similarity were performed online using BLAST on the NCBI website.

2.11. Artemia lethality test

The *Artemia* lethality test was conducted according to Carneiro et al. [21]. H-3 and HMA were dissolved in artificial sea water (ASW) at a concentration of 200 μg mL^{-1}. The assay was performed boarding 24-well Linbro® plates in which each well contained 10 *Artemia* nauplii in a final volume of 2 mL. Lectin solution was added to the wells at final concentrations of 12.5, 25, 50 and 100 μg mL^{-1}. The experiment was performed in triplicate, and the negative control wells contained 2 mL of artificial seawater with 10 *Artemia* nauplii. After 24 h, the number of dead nauplii in each well was counted.

2.12. Agglutination of bacteria

Escherichia coli and *Staphylococcus aureus* were grown in nutrient broth at 37 °C for 24 h, harvested by centrifugation at 2000 × *g* for 10 min, washed three times with TBS, suspended in TBS containing formaldehyde 4%, kept for 16 h at 4 °C, washed three times with TBS, and, finally, suspended in the same buffer. Bacterial count was calculated by the serial dilution method, and A_{625} was maintained around 1.0. Bacterial agglutination was tested by mixing 50 μL of HMA and H-3 (1 mg mL^{-1}) to an equal volume of bacterial suspension. Results were observed under a light microscope after incubation for 2 h [22].

2.13. Antioxidant activity

2.13.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was conducted according to Blois et al. [23], with some modifications. HMA and H-3 were solubilized in four different concentrations (12.5, 25, 50 and 100 μg mL^{-1}). The absorbance of sample, blank sample, and control was measured at 517 nm, after 30 min incubation in the dark at room temperature, using a Biochrom Asys UVM340 microplate reader (Cambridge, UK). The sample consisted of a mixture of 2.5 mL DPPH methanolic solution (0.16 mM) with 0.5 mL of HMA and H-3. The blank sample consisted of 0.5 mL of HMA and H-3 and 2.5 mL MeOH, while the control contained 3 mL DPPH methanolic solution (0.16 mM) only. The percentage of DPPH scavenging activity was calculated using the expression below:

$$\text{Scavenging effect (\%)} = \left[1 - \frac{\left(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample blank}}\right)}{\text{Abs}_{\text{control}}}\right] \times 100\%$$

2.13.2. β-Carotene bleaching assay

The method described by Chew et al. [24] was used for the inhibition of β-carotene oxidation. HMA and H-3 were solubilized in four different concentrations (12.5, 25, 50 and 100 μg mL^{-1}). Linoleic acid and Tween 40 were added to the β-carotene solution in chloroform (100 μg mL^{-1}). The chloroform was then evaporated, and oxygen-saturated ultrapure water was added to the residue. The β-carotene/linoleic acid emulsion was shaken vigorously, and aliquots of this emulsion were added to HMA and H-3 at different concentrations. The absorbance was read at 470 nm immediately after the emulsion was prepared ($\text{Abs}_{\text{initial}}$) and after 1 h of incubation in a water bath at 50 °C ($\text{Abs}_{1\text{h}}$). The percentage AOA was calculated with the following formula:

$$\text{Antioxidant activity (\%)} = \left(\frac{\text{Abs}_{1\ \text{h}}}{\text{Abs}_{\text{initial}}}\right) \times 100\%$$

2.13.3. Ferrous ion chelating assay

Ferrous ion chelating activity was determined according Wang et al. [25]. HMA and H-3 were solubilized in four different concentrations (12.5, 25, 50 and 100 μg mL^{-1}). The absorbance of sample, blank sample and control was measured at 562 nm after 10 min incubation at room temperature, using a Biochrom Asys UVM340 microplate reader (Cambridge, UK). The sample consisted of distilled water, 2 mM ferrous chloride ($FeCl_2$), 5 Mm ferrozine and HMA and H-3 at different concentrations. Distilled water was used for both blank sample and control, instead of ferrozine and HMA and H-3, respectively. The percentage of ferrous ion chelating activity was calculated using the expression below:

$$\text{Ferrous ion chelating activity (\%)} = \frac{\left[\text{Abs}_{\text{control}} - \left(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample blank}}\right)\right]}{\text{Abs}_{\text{control}}} \times 100\%$$

Table 1
Purification yield of HMA. [a] Minimal active concentration.

Fraction	Titer (HU mL^{-1})	Total protein (mg)	Specific activity (HU mg^{-1})	Total activity	Yield (%)	Purification fold (x)	[a]MAC (μg mL^{-1})
Crude extract	128	768	36	7708	100	1	27.89
HMA	64	14	256	3648	47	7	3.9

3. Results

3.1. Purification of HMA-1

A purple lectin was purified from *H. manglaris* by the combination of hydrophobic interaction chromatography (HIC) and ion exchange chromatography (IEC) (Fig. 1). The lectin was purified 7.1 times and represents 48% of the total hemagglutinating activity present in the crude extract (Table 1).

3.2. Molecular mass, sugar content and absorbance spectrum

On SDS PAGE, in the presence and absence of reducing agents, HMA showed two main bands: one broad band (α chain) and one slight band (β chain) with *Mr* 22,000 and *Mr* 15,000, respectively (Fig. 2A). On native conditions, HMA showed *Mr* 60,000 on N-PAGE (Fig. 2B) and a sharp and symmetrical peak of *Mr* 55,000 on size exclusion chromatography (Fig. 3).

HMA is a glycoprotein with 7% of neutral carbohydrate, as indicated by the phenol-sulfuric acid assay. After testing with PAS, HMA showed positive coloration (Fig. 2C).

Wave scan of HMA showed three absorbance peaks at 216, 280 and 660 nm, indicating the presence of a purple chromophore linked to the lectin (data not shown).

3.3. Hemagglutination and inhibition of HMA

HMA was able to agglutinate rabbit erythrocytes, showing marked preference by trypsin-treated erythrocytes, but it could not agglutinate human erythrocytes (Table 2). Hemagglutinating activity was only inhibited by thyroglobulin at minimum inhibitory concentration (MIC) of 0.5 mg mL^{-1}.

3.4. Effects of pH, temperature and divalent cations on the hemagglutinating activity of HMA

The hemagglutinating activity of HMA was stable up to 60 °C, with total loss of activity after heating to 80 °C. HMA activity was stable between pH 8 and 9. The activity decreases at acidic pH and is abolished at pH 4.0 (data not shown). The presence of $CaCl_2$, $MnCl_2$, $MgCl_2$ and EDTA did not affect the hemagglutinating activity of HMA.

3.5. Molecular mass and sulfhydryl groups determination by mass spectrometry

Reverse phase chromatography (RPC) on C-8 column leads to separation of HMA chains (Fig. 4). The first peak (β-chain) showed a molecular mass of 14,201 ± 2 Da (Fig. 5A), and the second peak (α-chain) showed a molecular mass of 25,262 ± 2 Da (Fig. 5B). Mass differences of 162 Da (hexose) were observed around the α-chain value of molecular mass, suggesting the presence of glycoforms.

Interestingly, when the second peak was infused onto mass spectrometer, a strong ionization signal in the low mass zone was observed. This signal corresponded to ion at *m/z* $[M+H]^{1+}$ 581.44, most likely because of a weak linkage between chromophore and α-chain (Fig. 5C). To identify the structural constituents of the chromophore, ion at *m/z* $[M+H]^{1+}$ 581.44 was selected and fragmented (Fig. 5D). The fragmentation pattern suggests the presence of different chemical groups formed by carbon, nitrogen, oxygen and hydrogen in the chromophore's structure.

Four half-cysteines were found in the HMA α-chain, whereas two half-cysteines were found in the HMA β-chain. No free sulfhydryl groups were identified (data not shown).

Table 2
Hemagglutinating activity of HMA. Hemagglutination was expressed in titer (HU mL^{-1}).

Blood type	Native	Enzymatic treatment	
		Papain	Trypsin
Human A	–	–	–
Human B	–	–	–
Human O	–	–	–
Rabbit	–	8	32

3.6. N-terminal and amino acid sequencing

N-terminal sequencing analyses of HMA were initially left out because HMA has two polypeptide chains. Thus, after RPC, HMA chains were separately submitted to *N*-terminal sequencing. The α-chain showed a singular *N*-terminal, APXAPSIENSFQAXV, while the β-chain could not be sequenced by Edman degradation, indicating that the *N*-terminal appears to be blocked.

Tryptic peptides of HMA showed no similarity to any known protein. Table 3 summarizes the amino acid sequences of peptides obtained by LC–MS/MS.

3.7. Artemia lethality test and agglutination of bacteria

In the *Artemia* lethality test, HMA and H-3 presented low toxicity levels. H-3 killed 7%, 10%, 10% and 13% of the nauplii at concentrations of 12.5 μg mL^{-1}, 25 μg mL^{-1}, 50 μg mL^{-1} and 100 μg mL^{-1}. HMA did not kill any *Artemia* nauplii at the same concentrations.

Neither H-3 nor HMA was able to agglutinate bacterial cells.

3.8. Antioxidant activity

H-3 and HMA showed no capacity to scavenge free radicals, as determined by DPPH. However, both lectins showed a high ability to protect β-carotene and moderate the ferrous ion chelating activity. In the β-carotene bleaching assay, H-3 presented 86.69 ± 0.17%, 89.29 ± 0.16%, 91.22 ± 0.14% and 92.54 ± 0.29% of activity at concentrations of 12.5 μg mL^{-1}, 25 μg mL^{-1}, 50 μg mL^{-1} and 100 μg mL^{-1}, respectively, whereas HMA showed 91.07 ± 0.1%, 93.97 ± 0.2%, 94.88 ± 0.08% and 96.71 ± 0.32% of activity at the same respective concentrations (Fig. 6A). The calculated IC_{50} were 10.569 ± 0.055 μg mL^{-1} and 9 ± 0.037 μg mL^{-1} for H-3 and HMA, respectively. Both activities presented a dose-dependent response.

H-3 presented 12.4 ± 0.2%, 16 ± 0.2%, 17 ± 0.4% and 20.5 ± 0.1% of ferrous ion chelating activity at concentrations of 12.5 μg mL^{-1}, 25 μg mL^{-1}, 50 μg mL^{-1} and 100 μg mL^{-1}, whereas HMA showed 12.4 ± 0.3%, 13.6 ± 0.2%, 20.6 ± 0.2% and 22.5 ± 0.2% of activity at the same respective concentrations (Fig. 6B). The calculated IC_{50s} were 0.460 ± 0.005 mg mL^{-1} and 0.322 ± 0.004 mg mL^{-1} for H-3 and HMA, respectively. Also, both activities occurred in a dose-dependent manner.

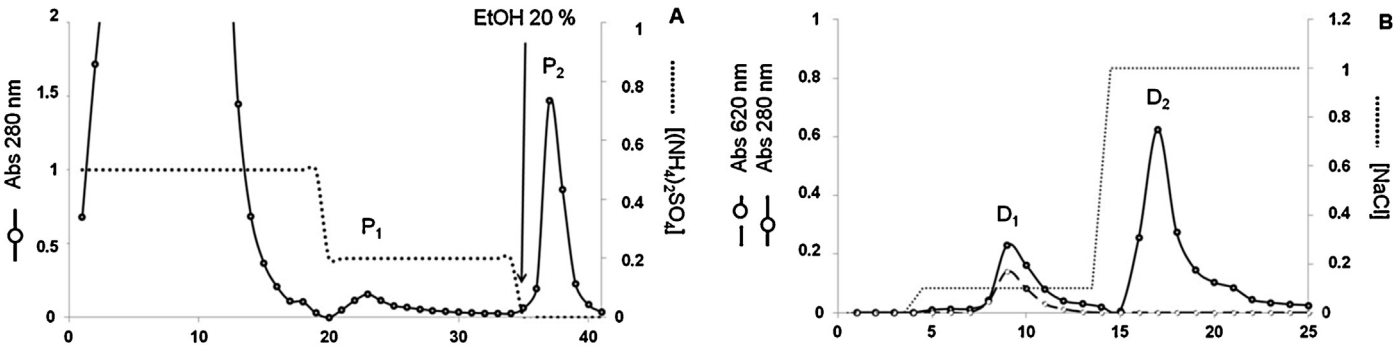

Fig. 1. Purification of HMA. (A) Approximately 40 mL of crude extract containing $(NH_4)_2SO_4$ 500 mM were applied onto a Phenyl-Sepharose matrix. The column was equilibrated and washed with acetate buffer, pH 5.5, containing $(NH_4)_2SO_4$ 500 mM at a flow rate of 2 mL min^{-1}. Active fractions (P_2) were eluted with ethanol 20%, as indicated by arrow. (B) Phenyl Sepharose fraction P_2 was dialyzed, freeze-dried and loaded onto a DEAE-Sephacel column equilibrated with Tris buffer, pH 7.6. Active fractions (D_1) were termed HMA. Chromatographies were monitored at 280 nm and 660 nm.

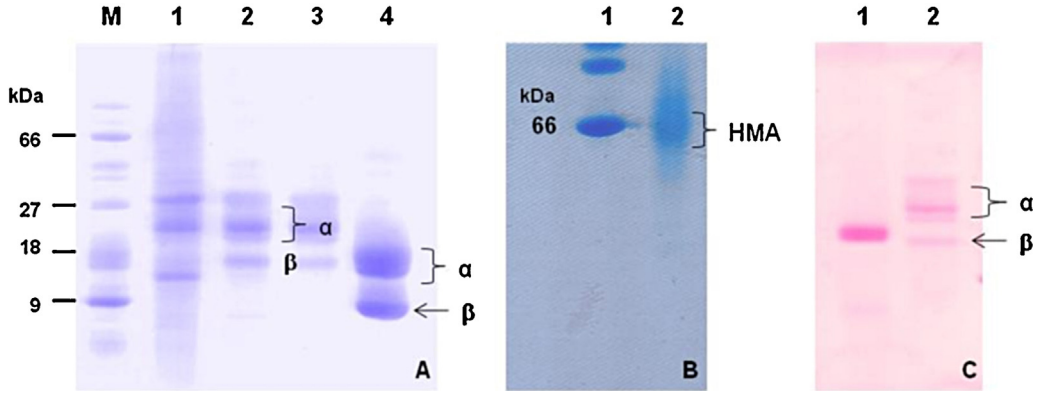

Fig. 2. Electrophoresis profile of HMA. (A) **SDS PAGE 15%. (M)** Molecular marker; **(1)** Crude extract of *H. manglaris*; Purified HMA in the absence **(2)** and presence **(3)** of 2-mercaptoethanol; **(4)** Purified H-3. (B) **Native PAGE. (1)** BSA; **(2)** Native HMA. (C) **SDS PAGE stained with PAS. (1)** H-3; **(2)** HMA.

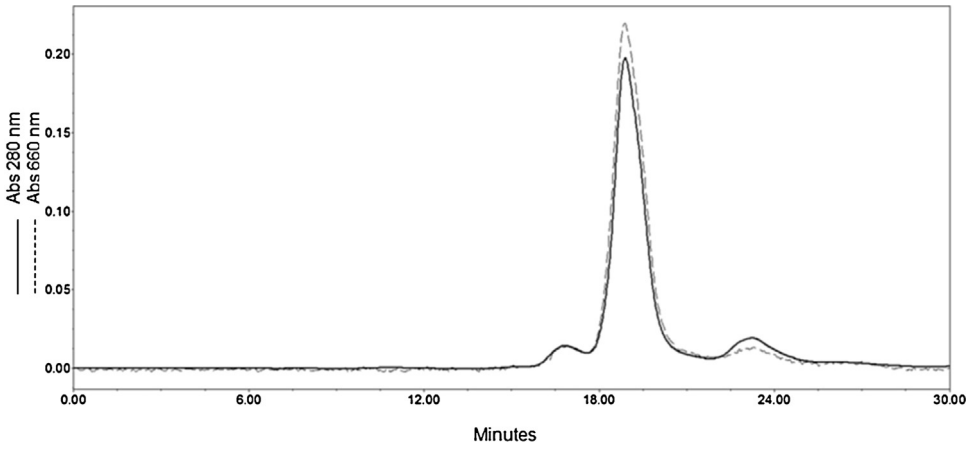

Fig. 3. Size exclusion profile of HMA in BioSuite 250.5 μm HR SEC colunm. Absorbance was monitored at 280 nm and 660 nm.

Table 3
Peptides sequenced from the tryptic digests of HMA using tandem mass spectrometry.

Peptide	*m/z*	Sequence	Mass		Δ (Da)
			Observe	Calculated	
α-T1	727.90	QYSGV[L/I]F[L/I]KSGQK	1453.79	1453.79	0.00
α-T2	609.72	QNGWDASDPTK	1217.42	1271.53	0.11
α-T3	850.30	SCSTTPPQWGFY[L/I]R	1698.58	1698.78	0.20
α-T4	597.57	KGNVTGD[L/I]PSFGVGEDAK	1789.70	1789.88	0.18
α-T5	646.22	QNGWDDQEATK	1290.44	1290.58	0.14
β-T1	539.26	AP[L/I] [L/I]SDSSCK	1076.51	1076.51	0.00
β-T2	651.85	[L/I]SD[L/I]DS[L/I] [L/I]AEVK	1301.69	1301.70	0.01
β-T3	681.83	P[L/I]A[L/I]SDSSQAFVK	1361.64	1361.71	0.07
β-T4	640.23	SSGTSYQ[L/I]PPSR	1278.45	1278.62	0.17
β-T5	572.27	FSNYVSW[L/I]K	1142.53	1142.57	0.04

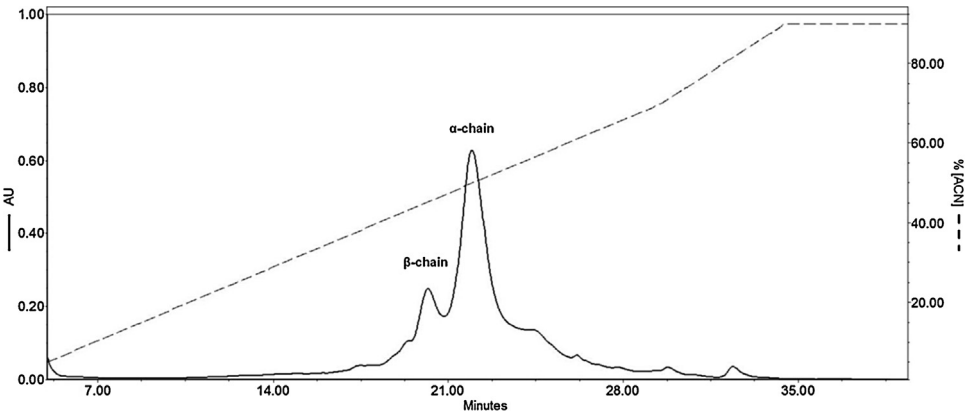

Fig. 4. Reverse phase chromatography of HMA. Retained fractions were recovered by elution with gradient of ACN (5–90%). The chromatography was monitored at 280 nm.

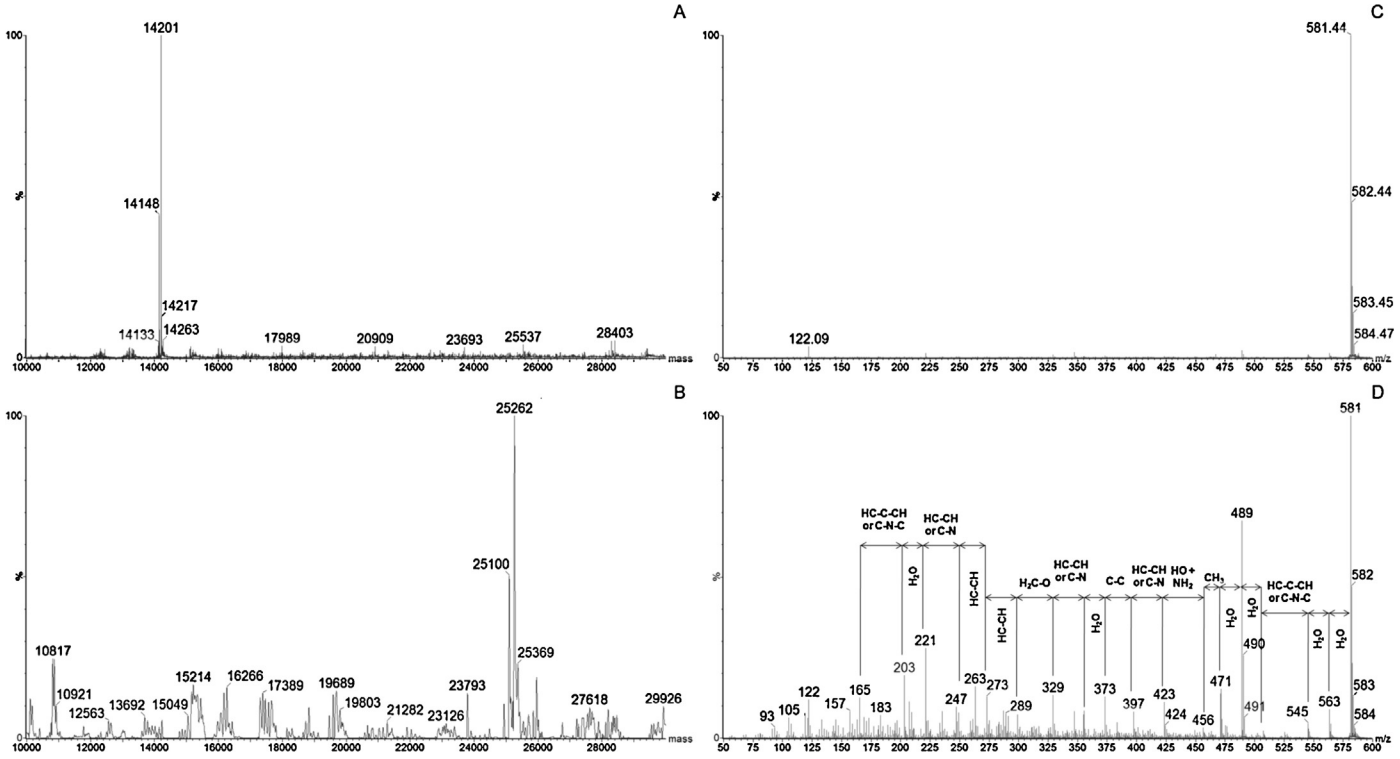

Fig. 5. Molecular mass determination of HMA. (A) Deconvoluted mass spectra of HMA β-chain after RPC. (B) Deconvoluted mass spectra of HMA α-chain after RPC. (C) Selected ion at *m/z* $[M+H]^{1+}$ 581.44. (D) Top-down fragmentation of the ion at *m/z* $[M+H]^{1+}$ 581.44. Fragments reveal the presence of organic compounds.

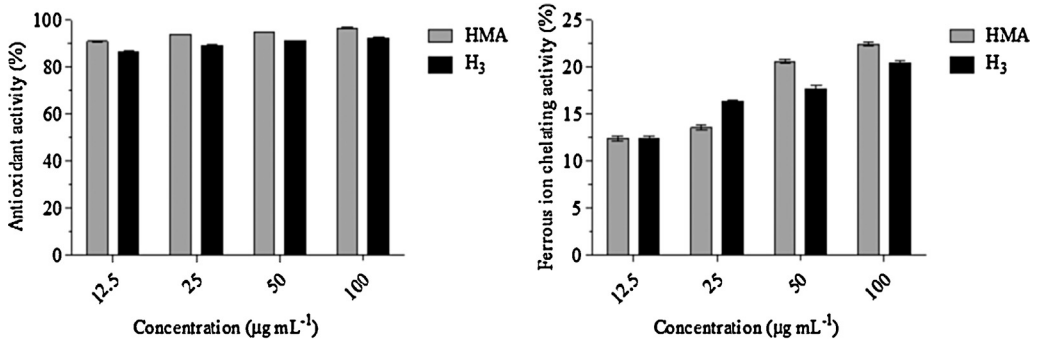

Figure 6. Antioxidant activity of H-3 and HMA. (A) **β-Carotene bleaching assay.** H-3 and HMA showed IC50 values of $10.569 \pm 0.055\ \mu g\ mL^{-1}$ and $9 \pm 0.037\ \mu g\ mL^{-1}$, respectively. (B) **Ferrous ion chelating activity.** H-3 and HMA showed IC50 values of $0.460 \pm 0.005\ mg\ mL^{-1}$ and $0.322 \pm 0.004\ mg\ mL^{-1}$, respectively.

4. Discussion

Lectins with natural chromophores could be interesting biotechnological tools. Therefore, having previously reported the purification of H-3, we decided to search for similar lectins. Proteins with this characteristic have been studied for their possible utilization as fluorescent probes [26]. For example, the green fluorescent protein isolated from *Aequorea victoria* (GFP) has been used in medicine, cell biology and molecular biology.

In another tropical sponge, *H. manglaris*, we have now found a lectin similar to H-3. Termed HMA, this lectin was successfully purified using a combination of HIC and IEC, similar to the purification of *Holothuria scabra* [27] and *H. caerulea* [11]. The high yield obtained from this purification process is unusual, but it is similar to that achieved in the purification of H-3 [11].

When exposed to variations of temperature and pH, HMA behaves in a manner similar to that of other sponge lectins that are often stable up to temperatures of 70 °C and in basic-neutral pH [28,29]. Basic-neutral pH represents the natural environment of these animals, and the relative thermal stability of sponge lectins mainly results from the presence of disulfide bonds in their structure, a common factor in sponge lectins [30,31]. Indeed, MS data revealed the presence of cysteines in HMA. All cysteines found in HMA appear be involved in intrachain disulfide bonds. H-3 presented the same amount of cysteines as HMA [11].

HMA is not a metalloprotein, such as lectins isolated from *Cinachyrella* and *Haliclona* species [7,11,29,32].

A few lectins isolated from marine sponges have been shown to recognize only complex carbohydrates [21,33]. HMA was only inhibited by thyroglobulin, a glycoprotein rich in residues of GlcNAc, mannose and GalNAc in the terminal position. On the other hand, H-3 was inhibited by GalNAc. Although GalNAc did not affect the hemagglutinating activity of HMA, it is possible that HMA recognizes cores containing GalNAc in the terminal position of tyroglobulin.

Like H-3, HMA is composed of two distinct polypeptide chains. In solution, HMA is likely a mixture of two β-chains and one α-chain, glycosylated or not. Therefore, HMA activity is a heterotrimer, an unusual formation in sponge lectins that typically consist of dimers or tetramers linked by weak interactions or disulfide bonds [7,26,34].

The molecular mass of HMA, as determined by MS, is in reasonable agreement with the mass estimated by electrophoresis. Divergences are sometimes observed between mass determined by MS and mass estimated by SDS-PAGE. These divergences result from intrinsic characteristics of the protein, such as internal disulfide bonds, glycosylation, or phosphorylation. Eventually, these characteristics modify the migration of a protein in electrophoresis.

PAS staining and Dubois assay indicated that HMA is a glycoprotein, and these data were confirmed by MS analysis. Several sponge lectins are glycoproteins [12,28,34]. Although it was possible to observe some hexose residues in the α-chain, we were not able to identify the glycan attached to HMA.

Unsurprisingly, a molecule of 581 Da was found when the HMA α-chain was analyzed by MS. This molecule is probably a chromophore attached to the HMA α-chain. Chromophore-581 found in HMA is an organic molecule much like the phycobilins found in stromata of algal cells. However, unlike phycobilins that are linked to their apoproteins by a thiol ester bond, chromophore-581 appears to be weakly linked to HMA, as inferred from its easy separation from HMA without chemical treatment.

H-3 also has a chromophore linked to its α-chain by weak interactions, but in H-3, the chromophore has a molecular mass of 597 Da, corresponding to one more oxygen molecule that chromophore-581. In phycobilins, a simple modification in chemical structure, *e.g.*, the presence/absence of one oxygen molecule or translocation of a double bond, can define the coloration of the chromophore [35]. For instance, the structural difference between phycoerythrobilin and phycocyanobilin is the position of a double bond, while the difference between their absorbances is tremendous. Phycoerythrobilin absorbs at 530 nm and emits red coloration, while phycocyanobilin has an absorption peak at 600 nm and emits blue coloration [36]. Thus, it is possible that the absence of a molecule of oxygen in chromophore-581, in comparison to chromophore-597, is sufficient to explain the difference of light absorption between the two chromophores (660 nm and 620 nm, respectively).

Although HMA and chromophore-581 being linked through weak interactions, extreme conditions seem be necessary to its separation, i.e. high voltage in mass spectrometry analysis; high concentration of organic solvents. Moderate variations of pH (3–11) and temperature (0–70 °C) did not affect interaction, as noted by the incubation of HMA in this conditions and posterior measurement by wavescan. Also, β-mercaptoethanol, EDTA and urea did not affect interaction (data not shown).

Despite the presence of a chromophore in both H-3 and HMA, they do not share sequence similarity. Moreover, HMA showed no sequence similarity with any protein. Therefore, we were unable to determine the complete sequence of HMA by MS techniques.

Unlike most lectins isolated from marine invertebrates that seem be involved in defense response [22,37,38], neither HMA nor H-3 showed toxic effects against *Artemia* nauplii. These lectins were also unable to agglutinate bacterial cells. However, their antioxidant activity, especially on β-carotene protection, suggests the putative involvement these lectins in antioxidant protection of the sponges. Interestingly, other sponge lectins have not shown such antioxidant activity by the same methods [39], leading us to believe that this activity is mediated by the chromophores present in HMA and H-3.

Harrison and Cowden [40] suggest that sponge synthesizes pigments that act like photoprotectors to minimize the action from solar radiation that could cause damage to vital metabolic products essential to the animal. *H. caerulea* and *H. manglaris* are tropical sponges, and in their natural environment, they are constantly assaulted by the physical elements, including high levels of solar radiation. Accordingly, it is possible that lectins or other proteins containing chromophores are involved in photo-oxidation protection.

In conclusion, we have isolated a new lectin from the tropical sponge *H. manglaris*. Despite sharing some characteristics with H-3, HMA showed no sequence similarity with any protein. Thus, H-3 remains an orphan with respect to the family of lectins. We also observed that lectins containing-chromophores can protect β-carotene against oxidation; therefore, we speculate that, *in vivo*, these lectins could protect sponge metabolites that are essential for survival.

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References

[1] D.C. Kilpatrick, Handbook of Animal Lectins, Properties and Biomedical Applications, British Library, Eddinburg, 2002.

[2] G.R. Vasta, H. Ahmed, Animal Lectins: A Functional View, CRC Press, New York, NY, 2008.
[3] G.R. Vasta, Nat. Rev. Microbiol. 7 (2009) 424–438.
[4] T. Ogawa, M. Watanabe, T. Naganuma, K. Muramoto, J. Amino Acids. 2011 (2011) 20 (Article ID 838914).
[5] R.M. Moura, K.S. Aragão, A.A. Melo, R.F. Carneiro, C.B. Osorio, P.B. Luz, A.F. Queiroz, E.A. Santos, N.M. Alencar, B.S. Cavada, Fundam. Clin. Pharmacol. 27 (2012) 656–668.
[6] Y. Fujii, N. Dohmae, K. Takio, S.M. Kawsar, R. Matsumoto, I. Hasan, Y. Koide, R.A. Kanaly, H. Yasumitsu, Y. Ogawa, S. Sugawara, M. Hosono, K. Nitta, J. Hamako, T. Matsui, Y. Ozeki, J. Biol. Chem. 287 (2012) 44772–44783.
[7] T. Ueda, Y. Nakamura, C. Smith, B.A. Copits, A. Inoue, T. Ojima, S. Matsunaga, G.T. Swanson, R. Sakai, Glycobiology 4 (2012) 412–425.
[8] X.W. Wang, X.F. Zhao, J.X. Wang, J. Biol. Chem. 289 (2014) 2405–2414.
[9] P. Delatorre, B.A.M. Rocha, E.P. Souza, T.M. Oliveira, G.A. Bezerra, F.B.M.B. Moreno, B.T. Freitas, T. Santi-Gadelha, A.H. Sampaio, W. Azevedo, B.S. Cavada, BMC Struct. Biol. 7 (2007) 52.
[10] P. Delatorre, J.S. Silva-Filho, B.A.M. Rocha, T. Santi-Gadelha, R.B. Nóbrega, C.A.A. Gadelha, K.S. Nascimento, C.S. Nagano, A.H. Sampaio, B.S. Cavada, Biochimie 95 (2013) 1–7.
[11] R.F. Carneiro, A.A. Melo, A.S. Almeida, R.M. Moura, R.P. Chaves, B.L. Sousa, K.S. Nascimento, S. Saker-Sampaio, J.P.M.S. Lima, B.S. Cavada, C.S. Nagano, A.H. Sampaio, Int. J. Biochem. Cell Biol. 45 (2013) 2864–2873.
[12] D. Gundacker, S.P. Leys, H.C. Schröder, I.M. Müller, W.E.G. Müller, Glycobiology 11 (2001) 21–29.
[13] H. Kamiya, K. Muramoto, R.R. Goto, Bull. Jpn. Soc. Sci. Fish. 56 (1990) 1159.
[14] M. Wiens, S.I. Belikov, O.V. Kaluzhnaya, A. Krasko, H.C. Schroder, S.P. Ottsatadt, W.E.G. Muller, Dev. Genes Evol. 216 (2006) 229–242.
[15] M.M. Bradford, Anal. Biochem. 72 (1976) 248–534.
[16] A.H. Sampaio, D.J. Rogers, C.J. Barwell, Phytochemistry 48 (1998) 765–769.
[17] U.K. Laemmli, Nature 227 (1970) 680–683.
[18] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F.F. Smith, Anal. Chem. 28 (1956) 350–356.
[19] R.M. Zacharius, T.E. Zell, J.H. Morrison, J.J. Woodlock, Anal. Biochem. 30 (1969) 148–152.
[20] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, Nat. Protoc. 1 (2006) 2856–2861.
[21] R.F. Carneiro, A.A. Melo, F.E.P. Nascimento, C.A. Simplicio, K.S. Nascimento, B.A.M. Rocha, S. Saker-Sampaio, R.M. Moura, S.S. Mota, B.S. Cavada, C.S. Nagano, A.H. Sampaio, J. Mol. Recognit. 26 (2013) 51–58.
[22] A.A. Melo, R.F. Carneiro, W.M. Silva, R.M. Moura, G.C. Silva, O.V. Sousa, J.S. Saboya, K.S. Nascimento, S. Saker-Sampaio, C.S. Nagano, B.S. Cavada, A.H. Sampaio, Int. J. Biol. Macromol. 64 (2014) 435–442.
[23] M.S. Blois, Nature 181 (1956) 1199–1200.
[24] Y.L. Chew, Y.Y. Lim, M. Omar, K.S. Khoo, LWT—Food Sci. Technol. 41 (2008) 1067–1072.
[25] T. Wang, R. Jónsdóttir, G. Ólafsdóttir, Food Chem. 116 (2009) 240–248.
[26] P. Bamfield, M.G. Hutchings, Chromic Phenomena: Technological Application of Colour Chemistry, RSC Publishing, Cambridge, 2010.
[27] N.M. Gowda, U. Gowsami, M.I. Khan, Fish Shellfish Immunol. 24 (2008) 450–458.
[28] R.M. Moura, A.F.S. Queiroz, J.M.S.L.L. Fook, A.S.F. Dias, N.K. Monteiro, J.K. Ribeiro, G.E. Moura, L.L. Macedo, E.A. Santos, M.P. Sales, Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 145 (2006) 517–523.
[29] D.S. Medeiros, T.L. Medeiros, J.K.C. Ribeiro, N.K.V. Monteiro, L. Migliolo, A.F. Uchoa, I.M. Vasconcelos, A.S. Oliveira, M.P. Sales, E.A. Santos, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 155 (2010) 211–217.
[30] F. Buck, C.I. Luth, H. Bretting, Biochem. Biophys. Acta 1159 (1991) 1–8.
[31] F. Buck, C. Schulze, M. Breloer, K. Strupat, H. Bretting, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 121 (1998) 153–160.
[32] I. Pajic, Z. Kljajic, N. Dogovic, D. Sladic, Z. Juranic, M.J. Gasic, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 132 (2002) 213–221.
[33] C. Xiong, W. Li, H. Liu, W. Zhang, J. Dou, X. Bai, Y. Du, X. Ma, Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 143 (2006) 9–16.
[34] P.B. Miarrons, M.M. Fresno, J. Biol. Chem. 275 (2001) 29283–29289.
[35] L. Barsanti, P. Gualtieri, Algae: Antamomy, Biochemistry, and Biotechnology, CRC Press, Boca Raton, FL, 2006.
[36] D. Voet, J.G. Voet, Biochemistry, Wiley, New York, NY, 1995.
[37] C.A. Janeway, R. Medzhitov, Annu. Rev. Immunol. 20 (2002) 197–216.
[38] G.R. Vasta, H. Ahmed, E.W. Odom, Curr. Opin. Struct. Biol. 14 (2004) 617–630.
[39] R.R. Dresch, C.B. Lerner, B. Mothes, V.M.T. Trindade, A.T. Henriques, M.M. Vozari-Hampe, Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 161 (2012) 365–370.
[40] F.W. Harrison, R.R. Cowden, Aspects of Sponge Biology, Academic Press, New York, NY, 1976.