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HGA-2, a novel galactoside-binding lectin from the sea cucumber *Holothuria grisea* binds to bacterial cells

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ABSTRACT

A novel lectin, HGA-2, was isolated from the sea cucumber *Holothuria grisea*. The protein was isolated by a single chromatographic step using a column of Guar Gum as affinity. HGA-2 showed an apparent molecular mass of 17 kDa and 34 kDa under reducing and nonreducing conditions, respectively. The hemagglutinating activity was specific for rabbit erythrocytes, showing no activity for human blood A, B and O. Its hemagglutinating activity was inhibited by carbohydrates containing galactose, with higher affinity for GalNAc and glycoprotein porcine stomach mucin (PSM). HGA-2 was stable at pH 6–10, significantly declining at pH 5 and a temperature of 40 °C, with its activity being abolished at 100 °C. The HGA-2 protein was found to be Ca^{2+}-dependent; it was highly toxic against *Artemia* nauplii and able to recognize and agglutinate cells of *Escherichia coli*. Amino acid sequences of tryptic peptides of HGA-2 strongly suggest that HGA-2 is a member of the C-type lectin family.

1. Introduction

Lectins are a group of carbohydrate-binding proteins that can be found in a wide variety of organisms, such as viruses, bacteria, fungi, plants and both vertebrates and invertebrates [1].

In animals, a number of processes are mediated by lectins, including intracellular routing of glycoproteins, cell–cell adhesion and phagocytosis. Different roles are attributed to intracellular lectins, such as trafficking, sorting and targeting of maturing glycoproteins, while those of extracellular lectins include cell adhesion, cell signaling, glycoprotein clearance and pathogen recognition [2,3].

A few studies have reported on the role of lectins in invertebrates, suggesting that they represent a major component of the defense system and that, as such, they contribute in a significant manner to their innate immune system by recognizing invading microbes and pathogens [4]. Bacterial agglutination is a common feature of lectins in marine invertebrates, and both coelomocytes and pattern recognition receptors (PRRs), as mediators of immune response, have the ability to recognize and bind surface structures on external foreign entities [5].

The ability to identify nonself can be attributed to C-type lectins and other proteins from the group of PRRs, and a PRR event can initiate signaling cascades that induce a downstream immune response or immediate reactions, such as agglutination, preventing movement and dispersal of the pathogen through the host and enabling opsonization for phagocytosis and destruction [6]. Studies on immune response have shown that the main function the echinoderm immune system is opsonization and that phagocytosis and C-type lectins are important in their immune system [7,8].

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In comparison with vertebrates, in particular mammals, only limited information about lectins can be found for marine invertebrates. However, in recent years, an increasing number of lectins have been reported from the phyla porifera [9], cnidaria [10], mollusca [11], arthropoda [12], echinodermata [13] and tunicate [14]. However, considering their particular characteristics, the lectins of marine invertebrates appear to be a potential tool for biochemical and biomedical applications.

Thus far, lectins from echinoderms have been the most studied group of marine invertebrate lectins, mainly from the class Holothuroidae. Those lectins belong to species, such as *Stichopus japonicus*, SJL-I and II [15] and SPL-1 and 2 [16]; *Cucumaria echinata* with 4 lectins isolated, CEL-I, II, III and IV [17]; *Cucumaria japonica*, MBL-C [18]; *Holothuria scabra*, HSL [7]; *Apostichopus japonicus*, MBL-AJ [19] and *Holothuria grisea*, HGA [13].

In the present work, we have isolated and purified a novel galactoside-binding lectin from the sea cucumber *H. grisea*, named HGA-2, and further characterized and described its physicochemical properties and its biological applications relative to the toxicity toward *Artemia* sp. and the agglutination of *Escherichia coli* cells.

2. Materials and methods

2.1. Animal collection (specimen)

Holothuria (Halodeima) grisea Selenka, 1867 (Holothuriidae, Apidochirotida, Holothuroidae, Echinodermata) specimens were collected at Bitupitá Beach, Ceará, Brazil (3°01′08″ S, 41°08′13″ W), transported in ice to the laboratory, and stored at −20 °C. The specimens were eviscerated, and the body walls were cut into pieces and lyophilized. The freeze-dried material was ground to a fine powder and extracted (1:10, w/v) with 150 mM NaCl in 50 mM Tris–HCl buffer, pH 7.6 (TBS) and then centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant was kept at −20 °C for further use.

2.2. Purification of H. grisea agglutinin 2 (HGA-2)

H. grisea extract was applied to a column of Guar gum (8.0 cm × 2.0 cm), previously equilibrated with 25 mM $CaCl_2$ in TBS at a flow rate 0.5 mL min^{-1}. The unadsorbed fractions were washed with equilibrium buffer at a flow rate of 0.5 mL min^{-1} until absorbance at 280 nm was less than 0.02. Adsorbed fractions were eluted with 20 mM EDTA in TBS at a flow rate of 0.2 mL min^{-1} and then pooled, dialyzed against deionized water, freeze-dried and stored until further use. Protein content from all fractions was determined by the Bradford assay [20] using BSA as standard. Neutral carbohydrate content was evaluated according to the method of Dubois [21] with D-glucose as the reference substance.

2.3. Hemagglutination and inhibition assays

Human (A, B and O) and rabbit erythrocytes were washed six times with 150 mM NaCl. A final suspension of 3% (v/v) was prepared and treated with different enzymes, trypsin and papain (10 μg mL^{-1}) at 37 °C for 1 h. Enzyme-treated erythrocytes were washed six times with 150 mM NaCl before use.

Hemagglutination tests were performed in microtiter plates with V-bottom wells by the two-fold serial dilution method according to Debray et al. [22]. Fifty microliters of the lectin fractions were added to each well with an equal volume of rabbit and human (A, B and O) erythrocytes (3%, v/v) and incubated for 30 min at room temperature. One hemagglutinating unit (H.U.) was defined as the amount of lectin able to agglutinate and, hence, precipitate the erythrocytes in suspension after 30 min.

Carbohydrate binding specificity was evaluated by determining hemagglutinating activity (H.A.) using standard methods [23] in the presence of sugars and glycoproteins (D-galactose, D-glucose, D-mannose, D-fructose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, methyl-α-D-glucopiranoside, methyl-α-D-galactopiranoside, D-lactose, D-sucrose, porcine stomach mucin (PSM) and orosomucoid). An equal volume of the lectin solution was added to 25 μL of the two-fold serially diluted inhibitor solutions, and the plate was incubated for 1 h at room temperature. Twenty-five microliters of rabbit erythrocytes were added to each well and allowed to incubate for 30 min at room temperature. Results were expressed as the minimal sugar or glycoprotein concentration required to inhibit hemagglutinating doses of the lectin.

2.4. Effects of pH, temperature and $CaCl_2$ on hemagglutinating activity

The effect of pH on H.A. was evaluated by incubation of the lectin (1 mg mL^{-1}) in the selected buffers (100 mM sodium acetate, pH 4.0–5.0; 100 mM sodium citrate, pH 6.0; 100 mM Tris–HCl, pH 7.0–8.0; 100 mM glycine, pH 9.0–10.0), followed by determination of the H.A., as described above.

Thermal stability of HGA-2 was monitored in the range of 30–100 °C by incubation of the lectin (1 mg mL^{-1}) for 60 min at respective temperatures on a thermal block followed by determination of the H.A. toward rabbit erythrocytes.

To assess the effects of divalent ions on H.A., hemagglutination tests were performed after exhaustive dialysis of the lectin (1 mg mL^{-1}) against 20 mM EDTA in the presence and absence of $CaCl_2$ (25 mM) [9].

2.5. Effect of denaturant and reducing agents on hemagglutinating activity

The effect of urea and reducing agents was performed as described by Dresch et al. [24]. The effect of urea on lectin activity was assessed by the incubation of HGA-2 (1 mg mL^{-1}) with rabbit blood erythrocytes in the presence of 4 M urea for 30 min at 20 °C. The effect of reducing agents on HGA-2 activity was studied by incubating the lectin (1 mg mL^{-1}) in the presence of 5 mM of DTT for 30 min at 20 °C followed by an evaluation of H.A.

2.6. Molecular mass estimation and subunits determination

SDS-PAGE was conducted in the presence and absence of β-mercaptoethanol (0.1 M) to estimate the molecular mass and the homogeneity of the purified lectin [25]. Proteins were detected by staining with 0.1% Coomassie Brilliant Blue R-250. BSA (66 kDa), ConBr α-chain (25 kDa), ConBr β-chain (14 kDa) and ConBr γ-chain (12 kDa) were used as molecular weight markers.

2.7. Artemia lethality test

The *Artemia* lethality test was conducted according to Carneiro et al. [9]. *Artemia* cysts were hatched under constant lighting and strong aeration in artificial seawater (ASW) at 28 °C. The cysts were incubated in a polyethylene cylindroconical tube with 1 g cysts per liter of ASW. This hatching condition simulated *Artemia's* natural environment. After a period of 48 h, the aeration was halted, and the lighting was directed to the bottom of the hatching vessel. The phototropic nature of the nauplii caused their migration in the direction of the light toward the bottom of the tube, while the unhatched cysts floated. The nauplii were then collected and used for bioassays.

HGA-2 was dissolved in ASW at a concentration of 200 μg mL^{-1}. The assay was performed on 24-well Linbro® plates in which each

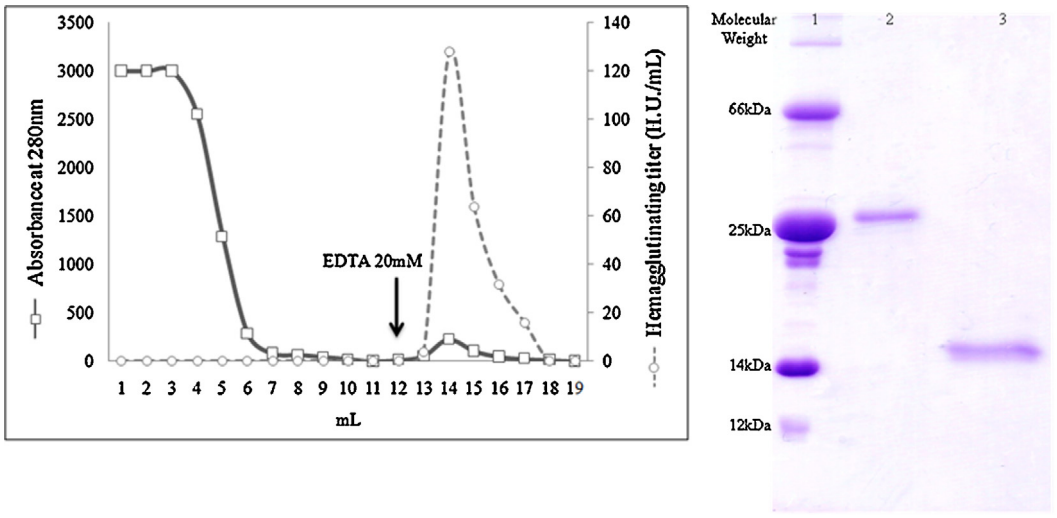

Fig. 1. Purification of lectin from the sea cucumber *H. grisea*. (A) The crude extract was applied to an affinity column of Guar gum. The elution was achieved with TBS-EDTA. The flow rate was adjusted at 0.2 mL min^{-1}. (B) SDS-PAGE of HGA-2. (1) Molecular weight markers (kDa): BSA (66), ConBrα-chain (25), ConBr β-chain (14) and ConBr γ-chain (12); (2) HGA-2 in the absence of β-mercaptoethanol. (B) HGA-2 in the presence of β-mercaptoethanol.

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 or 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. From these data, we calculated the percentage of dead at each concentration and the LC50 value by Probit analysis, as described by Finney [26].

2.8. Antibacterial activity

Antibacterial activity of HGA-2 was conducted under "*in vitro*" conditions by the agar adsorption technique adapted from Gowda et al. [7]. *E. coli* and *Staphylococcus aureus* were cultured in nutrient broth for 24 h, and for each test, the cultures were diluted, adjusting to A_{625} = 1.0. A sterile cotton bud was then dipped into the bacterium culture, and the inocula were spread uniformly on the surface of the agar media to produce a bacterial field. Subsequently, disks soaked in HGA-2 (1 mg mL^{-1}) in TBS-Ca^{2+} were applied in triplicate. A negative control disk (soaked in TBS-Ca^{2+} only) and a positive control disk (soaked in 5 μg ciprofloxacin; Laborclin) were used. The plates were incubated at 35 °C for 24 h. Inhibition halos bigger than 6 mm were considered evidence of antibacterial activity.

2.9. Agglutination of bacteria

E. coli and *S. aureus* were grown in nutrient broth at 37 °C for 24 h and harvested by centrifugation at 2000 × *g* for 10 min. They were washed three times with TBS, suspended in TBS containing formaldehyde 4%, and kept for 16 h at 4 °C. Finally, they were washed three times with TBS and then suspended in the same buffer. Bacterial count was calculated by the serial dilution method, and absorbance at 625 nm was maintained around 1.0. Bacterial agglutination was tested by mixing 50 μL of HGA-2 (1 mg mL^{-1}) to an equal volume of bacterial suspension. In another set, HGA-2 was incubated with 100 mM of D-galactose, as ligand protection, for 30 min and later mixed with equal volume of bacterial suspension. Results were observed under a light microscope after incubation for 2 h [10].

2.10. Amino acid sequencing by tandem mass spectrometry

SDS-PAGE was performed as described above. After staining, HGA-2 spots were excised, reduced with dithiothreitol (DTT) and carboxyamidomethylated with iodoacetamide (IAA) as described by Shevchenko et al. [27]. Carboxyamidomethylated spots were subjected to digestion with trypsin. Digestion was performed in ammonium bicarbonate 25 mM at 1:50 (w/w) (enzyme/substrate) and maintained at 37 °C for 18 h.

The digestion was stopped with 2 μL of 2% formic acid (FA). The samples were washed four times with 5% FA in 50% acetonitrile (ACN). The supernatants were collected and transferred to fresh tubes, pooled, vacuum-dried, solubilized in 20 μL of 0.1% FA, and centrifuged at 10,000 × *g* for 2 min.

Two microliters of the peptide solution were loaded onto a C-18 (0.075 mm × 100 mm) nanocolumn coupled to a nanoAcquity system. The column was equilibrated with 0.1% FA and eluted with a 10–85% 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 collision gas. All of the CID spectra were manually interpreted.

The sequence similarity of the peptides was evaluated online (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTp program from the National Center for Biotechnology Information. The alignment of the sequences of the CEL-IV and the sequenced peptides of HGA-2 was performed using EsPript 2.2 [28].

3. Results

3.1. Purification of HGA-2

The separation of HGA-2 from the crude extract was achieved by affinity chromatography in a column packed with cross-linked Guar gum, which separates proteins by their affinity to the galactose residues present in its structure. The bound protein was eluted from the column with the addition of 20 mM EDTA in TBS (Fig. 1A). The bound fractions were pooled, and hemagglutinating

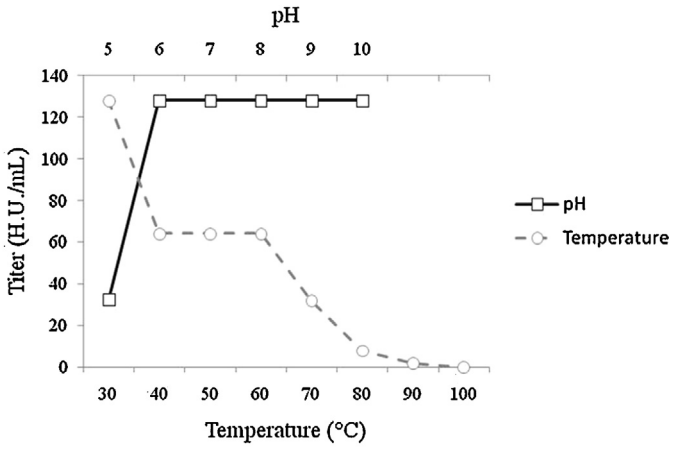

Fig. 2. Effects of temperature and pH on the hemagglutinating activity of HGA-2.

tests revealed that the separation of the hemagglutinating fraction was successful. In SDS-PAGE, under reducing conditions, HGA-2 appeared with one band of approximately 17 kDa and 34 kDa in the presence and absence of reducing agents, respectively (Fig. 1B). From a total of 38.4 mg of soluble proteins, 0.24 mg of HGA-2 was purified, representing a yield of 1.6%. HGA-2 has a specific activity of 3200 H.U. mg^{-1} and a minimal active concentration of 0.313 $\mu g\,mL^{-1}$.

3.2. Effects of pH, temperature and divalent ions on hemagglutinating activity

Exposure of HGA-2 to buffers with different pH values showed that the lectin was stable between pH 6 and 10, having a slight loss of activity on more acidic pH (Fig. 2). The activity of the lectin was stable until 30 °C, decreasing by half at 40 °C, and losing all of its activity only when heated to boiling temperature, 100 °C, for 1 h (Fig. 2). The lectin activity was completely abolished by the presence of 20 mM EDTA, but total H.A. was nearly restored after addition of 10 mM $CaCl_2$.

3.3. Effect of denaturant and reducing agents on hemagglutinating activity

HGA-2 exhibited different responses to treatment with urea and DTT. Treatment with 4 M urea decreased H.A. by 32-fold, while the incubation of the lectin with DTT showed no difference on H.A.

3.4. Hemagglutinating and inhibition assay

HGA-2 was shown to be specific to rabbit native and enzyme-treated erythrocytes to almost the same degree. The lectin was not able to agglutinate human (A, B and O) erythrocytes.

The results of sugar inhibition tests, using simple sugars and glycoproteins, are shown in Table 1. The results showed that HGA-2 exhibited preference for galactose and carbohydrates containing galactose residues. The lectin was inhibited by the mono- and oligosaccharides D-galactose, D-lactose, methyl-α-D-galactopiranoside, *N*-acetyl-D-galactosamine and the glycoprotein porcine stomach mucin (PSM).

3.5. Artemia lethality test

In the *Artemia* lethality test, HGA-2 presented a high mortality rate, killing 100% of the nauplii at concentrations of 100 and 50 $\mu g\,mL^{-1}$, 90% at 25 $\mu g\,mL^{-1}$ and 80% at 12.5 $\mu g\,mL^{-1}$ with a LC50 of less than 12.5 $\mu g\,mL^{-1}$.

Table 1
Inhibition of hemagglutinating activity of HGA-2.

Inhibitory compounds	MIC[a]
D-Galactose	12 mM
D-Glucose	NI
D-Mannose	NI
D-Fructose	NI
N-Acetyl-D-glucosamine	NI
N-Acetyl-D-galactosamine	6 mM
Methyl-α-D-glucopiranoside	NI
Methyl-α-D-galactopiranoside	12 mM
D-Lactose	12 mM
D-Sucrose	NI
Porcine stomach mucin (PSM)	1.25 mg mL^{-1}
Orosomucoid	NI

[a] Minimum inhibitory concentration; NI: sugar not inhibitory until a concentration of 100 mM.

3.6. Antibacterial activity

Assay of antibacterial activity with purified HGA-2 revealed that the lectin shows no antibacterial activity *in vitro* to Gram-positive and Gram-negative bacteria.

3.7. Agglutination of bacteria

As seen in Fig. 3, HGA-2 at a concentration of 100 $\mu g\,mL^{-1}$ was able to agglutinate only the Gram-negative bacteria *E. coli*, although when incubated for 30 min with 0.1 M D-galactose, the lectin was unable to agglutinate it. HGA-2 showed no agglutinating activity toward *S. aureus*, exhibiting preference for certain carbohydrates present on the Gram-negative bacteria cell wall. *Haliclona* sp. lectin was used as positive control for *S. aureus*, and as a negative control, TBS containing 20 mM of $CaCl_2$ was used, and no bacterial aggregates were observed.

3.8. Primary structure of HGA-2

The partial primary structure of HGA-2 was determined by *in situ* digestion of its electrophoretic bands with trypsin (Table 2). Tryptic peptides of HGA-2 showed similarity with C-type lectins, such as CEL-IV (Fig. 4), CEL-I (*C. echinata* lectins) and CTL-2 (*Necator americanus* lectin). These data strongly suggest that HGA-2 is a member of the C-type lectin super family.

4. Discussion

In the present study, we focused on the isolation, biochemical characterization and detection of biological activities toward *Artemia* and bacteria of a new galactoside-binding lectin (HGA-2) from the sea cucumber *H. grisea*. The lectin was successfully purified in a single step using affinity chromatography with the polysaccharide Guar gum as the affinity ligand of the chromatography stationary phase.

Guar gum is a galactomannan composed of a chain of β-(1→4)-linked D-mannopyranosyl residues having attached α-D-galactopyranosyl units linked α-(1→6) as single unit side chains [29,30]. Because HGA-2 is a galactoside-specific lectin and shows no affinity to mannose, we strongly believe that the galactose branches were responsible for purifying the lectin from the crude extract. Affinity chromatography is a common technique used for lectin purification based on its characteristic facile purification in a single step. Among sea cucumbers, affinity chromatography is frequently used as an important step for

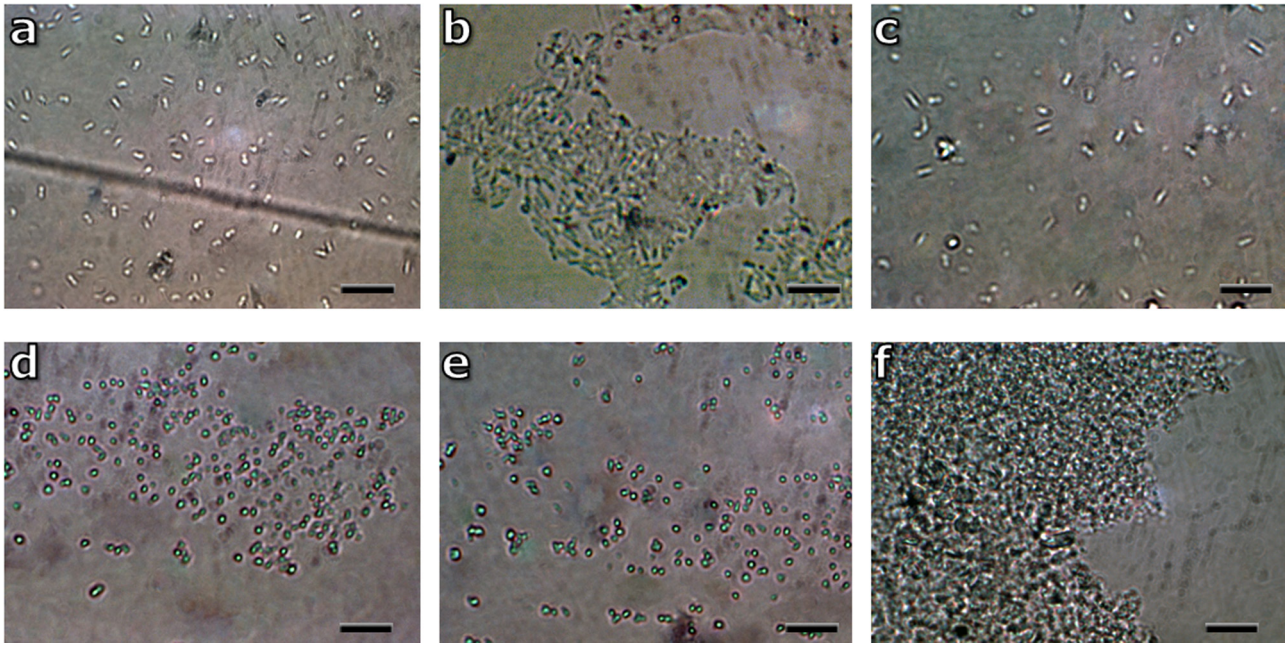

Fig. 3. Agglutination of bacteria by HGA-2. *Escherichia coli* incubated with TBS-Ca^{2+} (A), HGA-2 (B) and HGA-2 incubated with 0.1 M D-galactose (C). *Staphylococcus aureus* incubated with TBS-Ca^{2+} (D), HGA-2 (E) and the positive control, Halilectin (F). Scale bars indicate 4 μm.

Table 2
Peptides sequenced from the tryptic digests of HGA 2 using tandem mass spectrometry.

m/*z*	Amino acid sequence	Mass (Da)		Δ (Da)
		Observed	Calculated	
849.9366	[L/I][L/I]T[L/I]CPPQWTQSVR	1697.8575	1697.8923	0.03
371.2271	VVSDGHK	740.4386	740.3817	−0.06
696.7299	GA[L/I]AANYSEG[L/I]WADVHSNGR	2087.1663	2087.9819	−0.18
784.9406	F[L/I]NN[L/I]NFN[L/I]AQNF	1567.8656	1567.7783	−0.09
732.7021	DTCSEGWFGFHDTASCYR	2194.0828	2194.8474	−0.24

protein purification. Lectins from the sea cucumber *C. echinata* [17] were purified using lactosyl-Sepharose 4B as a first step, isolating four lectins, CEL-I, II, III and IV, from the crude extract. The four lectins were separated using a combination of size exclusion chromatography on Sephacryl S-200 and ion-exchange chromatography on Q-Sepharose. The MBL-AJ from the sea cucumber *A. japonicus* [19] was isolated by a combination of ion-exchange chromatography on DEAE TSK 650 M column and affinity chromatography on a mannan-Sepharose CL-4B column. SJL-I and II [15] from the sea cucumber *S. japonicus* were purified by a combination of affinity chromatography on lactosyl-Sepharose 4B and ion-exchange chromatography on Q-Sepharose, while SPL-1 and 2 [16] from the same species were purified by affinity chromatography on a porcine stomach mucin-conjugated agarose column followed by gel filtration on a Superose 6 column and ion-exchange chromatography on a HiTrap Q-FPLC. Different from those lectins, HGA from *H. grisea* and HSL from *H. scabra* did not involve affinity chromatography in their purification methods, using instead only a combination of size exclusion chromatography on a TSKgel G3000SWXL column and ion-exchange chromatography on a HITRAP DEAE FF column for HGA [13] and hydrophobic interaction chromatography on a Phenyl-Sepharose column for HSL [7].

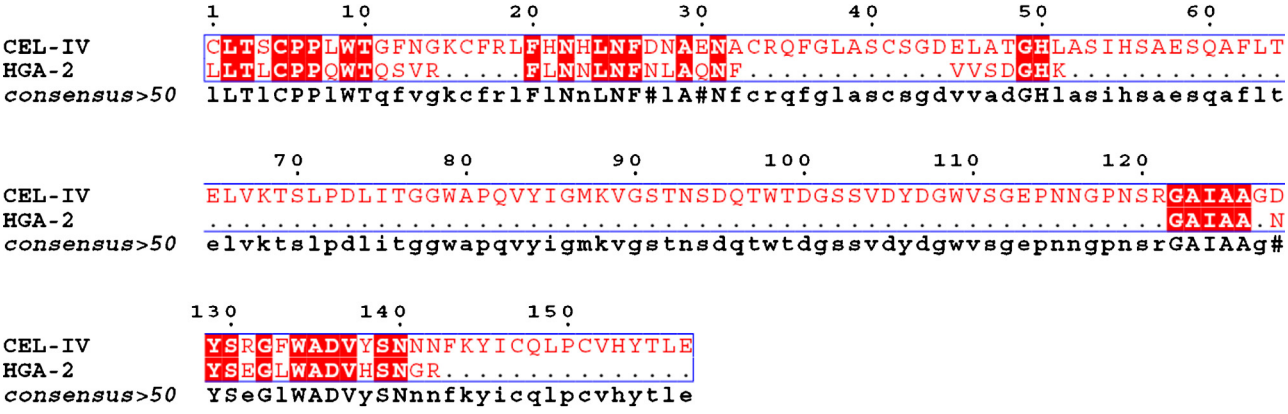

Fig. 4. Comparison of the amino acid sequences of HGA and CEL-IV.

The crude extract of *H. grisea* presented hemolytic activity toward rabbit and human erythrocytes. However, the purification protocol used in the present work was able to separate the hemagglutinating activity from the hemolytic activity in a single step. The presence of hemolysis is common in marine invertebrates and can be caused by a variety of molecules, including proteins. It was reported that *C. echinata* had the same hemolytic activity as that observed in *H. grisea* extract, and the activity was attributed to a lectin, CEL-III [17]. This protein has a pore-forming characteristic that enables it to cause hemolysis on the erythrocyte membrane [31]. The *Laetiporus sulphureus* lectin (LSL) is another lectin that presents hemolytic activity [32] and shares the same mechanism of action as that of CEL-III, namely, binding to the cell surface of carbohydrates with N-terminal β-trefoil lectin domains, followed by pore formation with C-terminal long β-strand-rich domains [33].

H.A. of HGA-2 was influenced by pH, temperature, presence of Ca^{2+} and addition of 4 M urea. Common features are shared by lectins isolated from sea cucumbers.

HGA-2 activity was pH-dependent. It was stable at pH 6.0–9.0, but decreased at pH 5.0. The buffer capacity of sea cucumber coelomic fluid is based on a carbonate–bicarbonate system. Sea cucumber pH ranges between 6.0 and 7.5, depending on the animal's physiological state [19], and bacterial injury or infestation of the animal results in an increase in coelomic fluid pH that could reach optimal value for exhibiting maximum lectin activity. Similar to HGA-2, lectins such as MBL-AJ [19], MBL-C [18], SJL-1 and 2, have pH stability varying from 7 to 9 with a decrease in activity in acidic pH. HSL, however, is stable through acidic, neutral and basic pH [7].

HGA-2 is a thermolabile protein with optimum activity at 30 °C, similar to other sea cucumber lectins, such as MBL-AJ, MBL-C and HGA [13,18,19]. HSL differs from HGA-2 and is highly thermo stability until 80 °C, having its activity extinguished only at 100 °C [7]. The persistence of activity up to 100 °C could be attributed to intrachain disulfide bonds, which are resistant to heating.

HGA-2 is a Ca^{2+}-dependent lectin, similar to other marine lectins. Ca^{2+} dependency is a common feature in the lectins of marine invertebrates, as reported in many marine sponges [34], corals [6], oysters [35], shrimps [36], sea cucumbers [37] and tunicates [38]. In the past, Ca^{2+} dependency was a defined characteristic from the C-type lectin family [39], although nowadays it is known that Ca^{2+} dependency is not the only characteristic of a C-type lectin (CTL). A CTL structure has a characteristic double-loop stabilized by two highly conserved disulfide bridges located at the bases of the loops, as well as a set of conserved hydrophobic and polar interactions. Such characteristics are a common sequence motif for the CTL family [40]. CEL-I and IV [37,41], MBL-AJ [19] and MBL-C [18] are known to be C-type lectins, while others, such as those isolated from *S. japonicus*, are Ca^{2+}-dependent, but owing to a few structural factors, they are not classified as C-type lectins, except SJL-I [42]. HSL and HGA are Ca^{2+}-independent and have a high molecular mass of 182 and 105 kDa, respectively, differing from the other sea cucumber lectins [7,13].

The inhibition of HGA-2 by D-galactose, methyl-α-D-galactopiranoside, D-lactose and GalNAc suggests that this is a galactoside-binding specific lectin, which is common in a variety of marine invertebrates. HGA-2 was also inhibited by the glycoprotein PSM, which contains *O*-linked carbohydrate structures sharing the core 1Galβ1–3GalNAc disaccharide [43], and the acetamide group present on GalNAc apparently resulted in an increase in specificity for GalNAc. The lectins from *C. echinata* are Gal/GalNAc-specific [17], as well as SPL-2 and SJL-I [16,42], while MBL-AJ and MBL-C [18,19], shows higher specificity to high mannan. The inhibition by PSM was also observed by the first lectin isolated from *H. grisea*, HGA, which has an affinity for *O*-glycans motifs as a result of the absence of inhibition by such monosaccharides as HSL [13].

HGA-2 has the ability to recognize and agglutinate rabbit erythrocytes and Gram-negative bacteria, including *E. coli*, but it was unable to agglutinate *S. aureus*. The addition of 0.1 M D-galactose could effectively inhibit agglutination by the lectin, indicating that the interaction of HGA-2 and *E. coli* was based on the recognition of galactose residues present in the lipopolysaccharide portion of the bacterial cell wall [44]. Peptidoglycans of Gram-positive bacteria, such as *S. aureus*, are mainly composed of repeating units of GlcNAc [44], which explains why HGA-2 was unable to agglutinate Gram-positive bacteria. In the absence of an acquired immune system, invertebrates rely on innate immunity to combat pathogens, and it is well known that lectins, especially C-type, play a major role in the innate immune system by acting as nonself recognition molecules [45].

Like HGA-2, other sea cucumbers lectins has ability of agglutinate and/or kill bacterial cells, such as HsL (*H. scabra* lectin) and CEL-III (*C. echinata* lectin) [7,46].

Studies reporting on the echinoderm immune system showed that coelomocytes are the central mediators of immune responses and that the agglutination of cells, especially bacteria, by lectins is a defense mechanism to prevent movement and dispersal of pathogens. This, in turn, enhances phagocytosis by mediating binding between coelomocyte surface and foreign body, thus exerting an opsonic function [8,47]. Many invertebrate lectins act as opsonins, including mannose-binding lectins and other lectins with different specificities [45]. Several studies focusing on the role of lectins in invertebrate immunity have been conducted with crustaceans, *e.g.*, lectins from the crab *Scylla serrata* and the shrimp *Penaeus japonicus*, which are involved in the opsonization process [48,49], while lectins of other crustaceans, such as *Macrobrachium rosenbergii*, *Liocarcinus depurator*, *Jasus novaehollandiae*, *Fenneropenaeus merguiensis* and *Parapenaeus longirostris*, are known for their ability to recognize and agglutinate bacterial cells [50–54]. The shrimps *Penaeus paulensis* and *P. californiensis* have lectins capable of agglutinating and opsonizing bacterial cells and enhancing phagocytosis by hemocytes [47,55]. Bacteria recognition and agglutination have also been reported in the cnidarians *Nemopilema nomurai* and *Acropora millepora*, the sea cucumber *H. scabra* and the horseshoe crab *Tachypleus tridentatus* [6,10,56]. HGA-2 agglutination was specific to Gram-negative bacteria, while HGA had no report of activity against bacterial cells. However, because HGA has different specificity for carbohydrates, we believe that it might possess a response to bacteria different from that of HGA-2. Lectins with different specificities are commonly reported in invertebrates, providing a means of efficient immune recognition equal to that provided by the immunoglobulins of vertebrates. Four different lectins are reported for the sea cucumber *C. echinata*. While these lectins share the same carbohydrate specificity, Gal/GalNAc, they differ in other features, such as molecular mass, oligomerization and structure [17]. Studies also reported the presence of four lectins in *S. japonicus* where all four have different specificities and structural characteristics [15,16]. Other marine invertebrates, such as the Japanese horseshoe crab *T. tridentatus*, have five lectins isolated from hemocytes and hemolymph plasma which are different in structure and specificity, suggesting a combinatorial method of pathogen recognition with different carbohydrates exposed on bacterial cell wall [56]. Sessile organisms, such as the coral *Sinularia lochmodes* [57] and the marine sponge *Haliclona caerulea* [9], have also had more than one lectin isolated, reported and characterized in terms of different carbohydrate specificity and structural features.

HGA-2 was highly toxic to *Artemia* nauplii in the instar II stage. The mortality rate appeared to increase with an increase in dose, indicating that the toxicity of HGA-2 to nauplii is dose-dependent.

Different marine invertebrates have been tested against *Artemia* sp., and it was observed that several of them were highly toxic to brine shrimp [58,59]. Among those invertebrates, sea cucumbers present a variety of biological activities with an emphasis on cytotoxic activity toward several cell lines and also toward brine shrimp by secondary metabolites and proteins [60–63]. Some marine invertebrate extracts were tested for toxicity against *Artemia* sp., human lung carcinoma and human colon carcinoma [58]. The results indicated that extracts of sponges, cnidarians, echinoderms and tunicate exhibited toxicity against *Artemia* sp. and human carcinoma. Collectively, these findings indicated that the *Artemia* lethality assay is useful for pre-testing the toxicity of potentially biologically active molecules. Lectins of the sea cucumber *C. echinata*, such as CEL-I and CEL-III, have demonstrated strong cytotoxic activities against MDCK, HeLa and XC, and MDCK, Vero, HeLa and human leukemia cells, respectively [62,63]. The similarity of HGA-2 to the GalNAc-specific lectins of the sea cucumber *C. echinata* and the strong cytotoxicity presented toward *Artemia* nauplii indicate a useful biomedical application of HGA-2 toward various cancer cells.

The results, together with the capacity to recognize Gram-negative bacteria, strongly suggest that HGA-2 might play an important role in physiological defense, as a nonself recognition molecule, and a potential role in the innate immune system of *H. grisea*, especially in the recognition of and protection against pathogens, as proposed for many other lectins of marine invertebrates [47].

Furthermore, we found that HGA-2 showed similarity of amino acid sequence with C-type lectins, suggesting that HGA-2 is a member of C-type lectin family. The highest similarity of sequence was observed when HGA-2 was compared with CEL-IV. CEL-IV is a homotetramer of C-type CRDs [17], while HGA-2 is a dimeric protein. Three dimensional structure of CEL-IV showed that the four subunits are further held together by interchain disulfide bonds among Cys1, Cys41 and Cys151. Dimmer units are linked by disulfide bonds between Cys41 and Cys151, while that Cys1 links the pairs of dimmers to former the tetrameric structure [41]. In HGA-2, Cys1 was substituted by Leu1 which prevents the formation of tetramers, such as could be confirmed by $[M+2H]^{2+}$ ion precursor of LLTLCPPQWTQSVR peptide at m/z 849.9366.

CEL-IV shows Gal/GalNAc specificity, but has an EPN motif (residues 113–115) that, generally, determine specificity of C-type lectins for mannose. The binding of CEL-IV to galactose is only possible by a modification in relative orientation of the carbohydrate. The bound galactose is inverted by nearly 180 °C in relation at others gal/galNAc binding C-type lectins with QPD motif [41]. Since that HGA-2 and CEL-IV shared similarity of primary structures, likely, HGA-2 is had EPN motif that recognize galactose.

In conclusion, we have isolated a novel lectin from the sea cucumber *H. grisea* which is specific to galactoside and the glycoprotein PSM. It is a homodimer of 34 kDa with subunits of 17 kDa linked by disulfide bonds in SDS-PAGE, thermoresistant to 90 °C and stable in neutral and basic pH. Although it agglutinates rabbit erythrocytes and the Gram-negative bacteria, *E. coli*, it showed no antibacterial activity against *E. coli* and *S. aureus*. Therefore, it is promising to explore the phagocytic and opsonic effect of HGA-2 in innate immunity of sea cucumbers at the molecular level. Furthermore, we found that HGA-2 shared similarity of primary structure with C-type lectins. We are presently working on solving the primary and quaternary structure of this lectin, as well as its role in the organism.

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