Anais da Academia Brasileira de Ciências (2015) 87(2): 973-984
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201520140399
www.scielo.br/aabc

Hemagglutinating/Hemolytic activities in extracts of marine invertebrates from the Brazilian coast and isolation of two lectins from the marine sponge *Cliona varians* and the sea cucumber *Holothuria grisea*

RANIERE M. MOURA[1], ARTHUR A. MELO[2], RÔMULO F. CARNEIRO[2], CÍCERA R.F. RODRIGUES[3], PLÍNIO DELATORRE[4], KYRIA S. NASCIMENTO[5], SILVANA SAKER-SAMPAIO[2], CELSO S. NAGANO[2], BENILDO S. CAVADA[5] and ALEXANDRE H. SAMPAIO[2]

[1]Universidade Potiguar, Escola de Medicina, Av. Sen. Salgado Filho, 1610, Lagoa Nova, 59054-000 Natal, RN, Brasil
[2]Laboratório de Bioquímica Marinha/BioMar-Lab, Departamento de Engenharia de Pesca, Universidade Federal do Ceará, Campus do Pici, Av. Humberto Monte, s/n, Bloco 871, 60440-970 Fortaleza, CE, Brasil
[3]Faculdade Leão Sampaio, Av. Maria Letícia Leite Pereira, s/n, 63040-405 Juazeiro do Norte, CE, Brasil
[4]Departamento de Biologia Molecular, Universidade Federal da Paraíba, Cidade Universitária, Av. Contorno da Cidade Universitária, 167, 58051-900 João Pessoa, PB, Brasil
[5]Laboratório de Moléculas Biologicamente Ativas/BioMol-Lab, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Campus do Pici, Av. Humberto Monte, s/n, Bloco 907, 60440-970 Fortaleza, CE, Brasil

Manuscript received on August 5, 2014; accepted for publication on November 28, 2014

ABSTRACT

Twenty species of marine invertebrates from the Brazilian coast were screened for hemagglutinating/hemolytic activity. In at least twelve tested species, hemagglutinating activity was different for different blood types, suggesting the presence of lectins. Extracts from four species showed hemolytic activity. Two new lectins were purified from the marine sponge *Cliona varians* (CvL-2) and sea cucumber *Holothuria grisea* (HGL). CvL-2 was able to agglutinate rabbit erythrocytes and was inhibited by galactosides. The hemagglutinating activity was optimal in pH neutral and temperatures below 70 °C. CvL-2 is a trimeric protein with subunits of 175 kDa. On the other hand, HGL showed both hemagglutinating and hemolytic activity in human and rabbit erythrocytes, but hemolysis could be inhibited by osmotic protection, and agglutination was inhibited by mucin. HGL was stable in pH values ranging from 4 to 10 and temperatures up to 90 °C. In electrophoresis and gel filtration, HGL was a monomeric protein with 15 kDa. CvL-2 and HGL showed different levels of toxicity to *Artemia* naplii. CvL-2 showed LC_{50} of 850.1 µg/mL, whereas HGL showed LC_{50} of 9.5 µg/mL.

Key words: purification, hemolysis, cytotoxicity, galactose.

Correspondence to: Alexandre Holanda Sampaio
E-mail: alexholandasampaio@gmail.com

INTRODUCTION

Marine biodiversity is the result of a long evolutionary history and extreme environmental conditions of pressure, light, oxygen, and temperature, giving rise to organisms with unique features (Ritchie et al. 2013). Marine life is much older than earthly life and has been evolving and diversifying longer (Burgess 2012). This amazing, but understudied, species diversity has provided a huge variety of molecules with biotechnological applications. Many of these compounds, which have already been isolated and studied, belong to marine invertebrates, such as sponges, cnidarians, mollusks, arthropods, echinoderms and tunicates (Rasjasa et al. 2011). Among these compounds are proteins (Pajic et al. 2002, Yoshida et al. 2007) and secondary metabolites (Kuramoto et al. 2004, Sipkema et al. 2005) with a wide range of biological activities.

Lectins are a group of carbohydrate-binding proteins found in a wide range of organisms, including viruses, bacteria, fungi, plants, and animals. Lectins are involved in various biological functions, such as host defense, cell–cell interaction and folding of glycoproteins (Kilpatrick 2002, Sharon and Lis 2004). Lectins from marine organisms are one of the promising candidates for useful therapeutic agents because carbohydrate structures, such as proteoglycans, glycoproteins, and glycolipids, have been implicated in certain cell types, and their physiological and pathological functions include host-pathogen interactions and cell-cell communication (Ogawa et al. 2011).

In particular, marine invertebrate lectins have attracted great interest due to their biotechnological properties. For instance, a lectin isolated from the sea cucumber *Cucumaria echinata* (CEL-III) was expressed in transgenic mosquitoes and impairs the development of the Malaria parasite (Yoshida et al. 2007). Lectins isolated from the marine sponges *Haliclona cratera* and *Craniella australiensis* displayed cytotoxic effect against malignant cells and mitogenic response towards BALB/c splenocytes, respectively (Pajic et al. 2002, Xiong et al. 2006).

The growing applications of lectins underscore the potential economic importance of the discovery of novel species for biotechnology. To date, the tropical sponge *Cliona varians* possesses one isolated lectin, CvL-1. CvL-1 is a galactose-binding lectin with cytotoxic effect on pathogenic bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus* (Moura et al. 2006). Furthermore, the sea cucumber *Holothurea grisea* possesses two isolated lectins: HGA, an anti-inflammatory lectin of 228 kDa, and HGA-2, a C-type lectin of 34 kDa (Moura et al. 2012, Melo et al. 2014). In the present work, we screened twenty Brazilian species of marine invertebrates for their hemagglutinating/hemolytic activity, and found two new lectins isolated from *C. varians* and *H. grisea*.

MATERIALS AND METHODS

Animal Collections

Marine sponges *Amphimedon viridis*, *Callyspongia* sp., *Haliclona caerulea*, *Haliclona melana*, and the sea urchin *Echinometra lucunter* were collected at Pacheco Beach, Caucaia, state of Ceará, Brazil.

Specimens of the marine sponge *Aplysina fulva*, cnidarians *Protopalythoa variabilis* and *Zoanthus sociatus*, mollusks *Ischinachiton pectinatus*, *Littorina ziczac*, *Pleuropoca* sp., *Turbinella laevigata*, and, *Voluta ebrea* and the sea cucumber *Holothuria arenicola* were collected at Paracuru Beach, São Gonçalo, Ceará State, Brazil.

Specimens of marine sponges *Aaptos* sp., *Haliclona manglaris* and *Spongorites* sp., were collected in Icaraí Beach, Amontada, state of Ceará, Brazil.

The decapods *Callinectes danae* were collected at Barra do Ceará Beach, Fortaleza, state of Ceará, Brazil.

Specimens of the marine sponge *Cliona varians* and the sea cucumber *Holothurea grisea* were collected at Santa Rita Beach, state of Rio Grande do Norte, Brazil.

The animals were transported in a thermal box at 4 °C and then stored at -20 °C until use. All collections were performed on sunny days, when water temperature was between 25 °C and 27 °C.

Preparation of Aqueous Extracts

Marine sponges were triturated in grail and homogenized in Tris buffer 50 mM, pH 7.6, containing NaCl 150 mM and $CaCl_2$ 20 mM (TBS/Ca^{2+}) at a ratio of 1:2 (w/v). The mixtures were filtered and centrifuged at 8000 x*g* for 20 min at 4 °C. Supernatants were stored at -20 °C until use.

The cnidarians were freeze-dried and triturated until a fine powder was obtained. The powders were homogenized in TBS/Ca^{2+} at a ratio of 1:2 (w/v). The mixtures were filtered and centrifuged at 8000 x*g* for 20 min at 4 °C. The supernatants were stored at -20 °C until use.

The mollusks and echinoderms *Holothurea grisea* and *H. arenicola* were cut into small pieces, triturated in mortar and pestle, homogenized in TBS/Ca^{2+} and filtered. After centrifugation, supernatants were stored at -20 °C until use.

Specimens of *Echinometer lucunter* were maintained on ice until dead for inanition. Then, peristomial membrane was cut around the mouth and the Aristotle's lantern was removed, and the celomatic plasma was collected by pipetting. The coelomic plasma was allowed to clot for 1h at 0 °C. The supernatants were stored at -20 °C until use.

The swimming crabs *C. danae* averaging 5 cm in length were maintained in ice. The hemolymph was collected from the cheliped using a sterile syringe, and allowed to clot for 1h at 0 °C. The supernatants were stored at -20 °C until use.

Crude extracts were used within three months after preparation.

Hemagglutinating/Hemolytic and Inhibition Assays

The hemagglutinating/hemolytic activities were assayed in microtiter V plates (Nunc, Denmark) as described by Sampaio et al. (1998). Native blood cells (Human ABO and rabbit erythrocytes) were treated with papain and trypsin. One hemagglutinating unit (HU) was defined as the reciprocal of the highest dilution giving positive hemagglutination. Hemolytic activity was examined by visual examination of lysis of the erythrocytes under conditions similar to those of the hemagglutination assay.

Purified lectins were submitted to inhibition assay as described by Sampaio et al. (1998). The following carbohydrates and glycoproteins were used: D-galactose, D-glucose, D-mannose, D-fructose, D-arabinose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, methyl-α-D-glucopyranoside, methyl-α-D-galactopyranoside, D-lactose, D-fructose, D-sucrose, D-lactulose, porcine stomach mucin (PSM), fetuin, tyroglobulin, ovomucoid, and orosomucoid. The initial concentrations were 100 mM or 5 mg/mL for sugars and glycoprotein, respectively.

In the assay using the lectin purified from *Holothuria griea*, erythrocytes were osmotically protected. Hemagglutinating activity was evaluated in the presence of dextrose 5.5%.

Purification of *Hotothuria grisea* Hemolytic Lectin (HGL)

Specimens of *H. grisea* were cut into small pieces. The pieces were washed in distilled water and freeze-dried. Pieces were then triturated in mortar and pestle and extracted at a ratio of 1:2 (w/v) with 0.05 M Tris–HCl buffer pH 7.6 containing NaCl 0.15 M (TBS) for 2h at room temperature. After centrifugation for 30 min at 8000 x*g* at 4 °C, the supernatant (crude extract) was partitioned with n-butanol 50% and ethanol 75% and then precipitated with acetone at 2.0 vol. The supernatant (F2) was dried in a vacuum concentrator and solubilized in a small volume of TBS. After centrifugation, F2 was

applied to a TSK gel G3000SWXL (1.28 × 30 cm) column coupled to an ÄKTA purifier system. The column was equilibrated and eluted with TBS at a flow rate of 1 mL/min. Chromatography was monitored at 280 nm, and 1 mL fractions were collected.

Purification of *Cliona varians* Lectins (CvL-1 and CvL-2)

C. varians crude extract was prepared as described above. The crude extract was precipitated with acetone at 1.0 vol. After centrifugation, the pellet was recovered with 0.05 M Tris buffer, pH 7.0, containing 1M $(NH_4)_2SO_4$ (Tris/$(NH_4)_2SO_4$). This CvL- -enriched fraction showed a higher level of hemagglutinating activity to rabbit trypsin-treated erythrocytes. CvL- -enriched fraction was then applied on Phenyl-Sepharose 6B column (1.0 x 6.0 cm) previously equilibrated with Tris/$(NH_4)_2SO_4$. The column was washed with the same buffer, and retained proteins were eluted with linear gradient of $(NH_4)_2SO_4$ (1 – 0M) in 0.05 M Tris, pH 7.0, at a flow rate of 2 mL/min. Fractions of 2 mL were manually collected. Active fractions were pooled, dialyzed against deionized water, and freeze-dried.

Freeze-dried proteins were solubilized with TBS/Ca^{2+} at a concentration of 5 mg/mL. After centrifugation, 1 mL was injected into a Sephacryl S-300 HR 16/60 column (1.6 x 60.0 cm) coupled to an ÄKTA purifier system. The column was equilibrated and eluted with TBS at a flow rate of 1 mL/min. Chromatography was monitored at 280 nm, and 3-mL fractions were collected.

Effects of pH, Temperature and Cations on Hemagglutinating/Hemolytic Activity of CvL-2 and HGL

The effect of pH, temperature and divalent ions on hemagglutinating/hemolytic activity was evaluated following Sampaio et al. (1998).

Molecular Mass Estimation of HGL and CvL-2

Sodium dodecyl sulfate polyacrylamide (10% and 15%) gel electrophoresis (SDS-PAGE), in the absence and presence of β-mercaptoethanol, was conducted as described by Laemmli (1970). The molecular mass of lectin and its subunits was estimated by comparing mobility of the bands with the following protein markers (kDa): Bovine serum albumin (Mr 67), Ovalbumin (Mr 43), Carbonic anhydrase (Mr 29) Ribonuclease (Mr 13.7) and Aprotinin (Mr 6.5). Proteins were detected by staining with 0.1% Coomassie brilliant blue R-250.

The native molecular mass of HGL was estimated by size exclusion chromatography on a TSKgel G3000SWXL (1.28 × 30 cm) column calibrated with the following protein markers (kDa): Conalbumin (Mr 74), Ovalbumin (Mr 43), Carbonic anhydrase (Mr 29), Ribonuclease (Mr 13.7) and Aprotinin (Mr 6.5).

The native molecular mass of CvL-2 was estimated by size exclusion chromatography on a Sephacryl S-300 HR 16/60 column (1.6 x 60.0 cm) calibrated with protein markers (Conalbumin, Ovalbumin, Carbonic anhydrase, Ribonuclease and Aprotinin). Native PAGE (N-PAGE) was also performed to estimate native molecular mass of CvL-2. The native molecular mass of CvL-2 on native PAGE was estimated by comparing mobility of the band with the following protein markers (kDa): Thyroglobulin (669), Ferritin (440), Catalase (232), Lactate dehydrogenase (140), and BSA (66).

Lethality Test in *Artemia*

Lethality test on *Artemia* nauplii was performed in accordance to pre-established methods (Carneiro et al. 2013).

Aqueous extracts of *C. varians* and *H. grisea* were dialyzed and freeze-dried. Extracts were prepared by homogenization with artificial sea water (ASW) at a concentration of 5 mg/mL. The purified lectins were solubilized in ASW at 1 mg/mL.

The tests were done on board 24-well Limbro plates such that each well contained 2 mL of artificial sea water (ASM) with 10 nauplii. Specific volumes of samples were added to each well to

obtain concentrations of 10, 25, 50 and 100 µg/mL for each sample. The experiments were performed in triplicate with control containing only 2 mL of ASW and 10 nauplii. Dead nauplii were counted after 24h. From these data, we calculated the percentage of deaths for each concentration and LC_{50} values were determined by probit analysis, as described by Finney (1971).

RESULTS

Lectin and Hemolytic Activity of Marine Invertebrate Aqueous Extracts

Twenty species of marine invertebrates were screened for their hemagglutinating/hemolytic activity, and the results are shown in Table I.

Eight of the nine species of sponges tested, exhibited hemagglutinating activity, and all species showed preference for some group/treatment of blood, suggesting the presence of lectin like molecules.

Species of the genus *Holothuria* showed both hemagglutinating and hemolytic activities. After agglutination, rapid hemolysis was observed. However, hemolysis was able to be halted by crude extract precipitation with 1 vol of acetone. In the extracts of the marine sponge *Amphimedon viridis* and the mollusk *Ischinachiton pectinatus*, hemolysis was also observed, but it could not be stopped.

Among the twenty species tested, we selected the two most abundant species, *C. varians* and *H. grisea*, for lectin purification and toxicity assay.

TABLE I

Screening of aqueous extracts of 20 marine invertebrate species from the Brazilian coast for hemagglutinating/ hemolytic activity. Hemagglutinating activity is described in titer. N-native erythrocytes; T-trypsin-treated erythrocytes; P-papain-treated erythrocytes; L-hemolytic activity observed.

	Rabbit			Human A type			Human B type			Human O type		
	N	T	P	N	T	P	N	T	P	N	T	P
PORIFERA												
Aaptos sp.	2^6	2^6	2^6	2^1	2^2	2^2	-	-	-	-	-	-
Amphimedon viridis	L	L	L	L	L	L	L	L	L	L	L	L
Aplysina fulva	2^{10}	2^{12}	2^{11}	2^8	2^8	2^8	2^8	2^8	2^8	2^8	2^8	2^8
Callyspongia sp.	2^8	2^8	2^8	L	L	L	L	L	L	L	L	L
Cliona varians	2^3	2^6	2^6	2^2	2^5	2^5	-	2^1	2^1	-	-	-
Haliclona caerulea	2^6	2^6	2^6	2^4	2^4	2^4	2^4	2^4	2^4	2^4	2^4	2^4
Haliclona manglaris	2^6	2^6	2^6	2^4	2^4	2^4	2^4	2^4	2^4	2^4	2^4	2^4
Haliclona melana	2^6	2^2	2^2	-	-	-	-	-	-	-	-	-
Spongorites sp.	2^6	2^6	2^6	2^1	2^1	2^1	-	-	-	-	-	-
CNIDARIA												
Protpalythoa variabilis	2^5	2^5	2^5	2^5	2^5	2^5	2^4	2^5	2^5	2^4	2^4	2^3
Zoanthus sociatus	-	-	-	-	-	-	-	-	-	-	-	-
MOLLUSCA												
Ischinachiton pectinatus	L	L	L	L	L	L	L	L	L	L	L	L
Littorina ziczac	-	-	-	-	-	-	-	-	-	-	-	-
Pleuroploca sp.	-	-	-	-	-	-	-	-	-	-	-	-
Turbinella laevigata	-	2^1	-	-	-	-	-	-	-	-	-	-
Voluta ebrea	-	-	-	-	-	-	-	-	-	-	-	-
CRUSTACEA												
Callinectes danae	2^4	2^5	2^5	2^3	2^4	2^4	2^3	2^4	2^4	2^2	2^2	2^2
ECHINODERMATA												
Echinometra lucunter	2^{10}	2^{10}	2^{10}	2^8	2^8	2^8	2^8	2^8	2^8	2^8	2^8	2^8
Holothurea arenicola	L	L	L	L	L	L	L	L	L	L	L	L
Holothurea grisea	L	L	L	L	L	L	L	L	L	L	L	L

Purification of Hemolytic Lectin from *Holothuria grisea*

The crude extract of *H. grisea* showed a higher level of hemagglutinating/hemolytic activity to all tested erythrocytes. After extract partition with n-butanol and ethanol, hemagglutinating/hemolytic activity was concentrated in Fraction 2 (F2). Thus, F2 was submitted to size-exclusion chromatography on TSKgel, where peak 1 corresponded to the pure hemolytic lectin HGL (Fig.1). This process resulted in purification of 35% with a 53% yield (Table II).

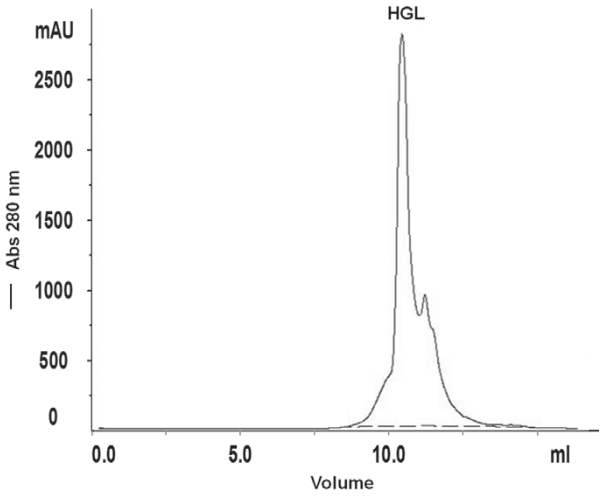

Figure 1 - Purification of HGL. TSKgel profile of F2 from *H. grisea*. Approximately 0.5 mg of F2 was applied to the column previously equilibrated with TBS pH 7.6, and fractions (1.0 mL each) were monitored at 280 nm. The hemolytic activity was detected in the presence of 3% rabbit erythrocytes.

Purification of the New Lectin from *Cliona varians*

The crude extract of *C. varians* was found to have strong hemagglutinating activity against trypsin-treated rabbit erythrocytes. CvL-2, a new lectin from *C. varians*, was purified by a combination

TABLE II
Purification processes of HGL. MHC – Minimum hemolytic concentration.

Fractions	Total protein (mg)	Titer	H.U./mg	Total activity	Purification	Yield (%)	MHC (µg/mL)
Crude extract	271.1	128	114.5	31040	1	100	8.75
F2	28.8	256	595.3	17152	5.2	55.3	1.68
TSK	4.1	2048	4079.7	16588.8	35.6	53.4	0.24

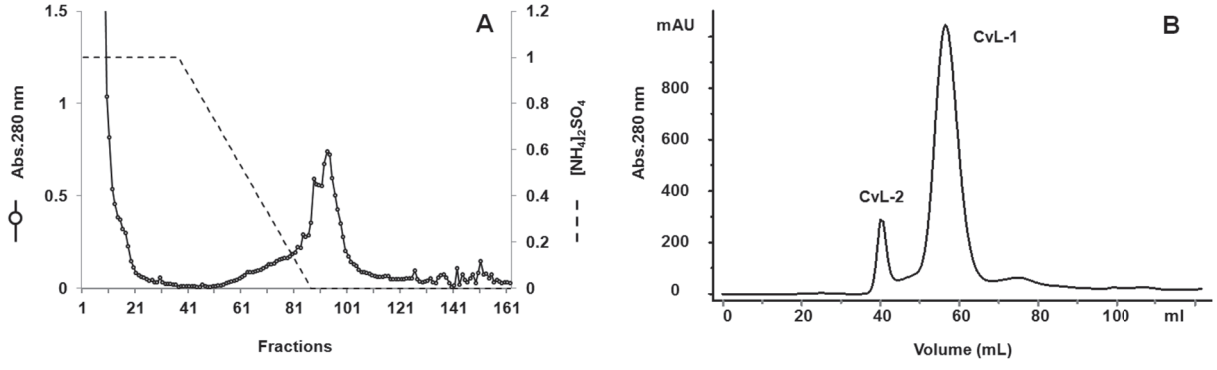

Figure 2 - Purification of CvL-2. (A) Phenyl Sepharose 6B chromatography profile. Approximately 100 mg of CvL-enriched fraction were applied to the column (1.0 × 6.0 cm) equilibrated with Tris/$(NH_4)_2SO_4$. The lectin was eluted with linear gradient of $(NH_4)_2SO_4$ (1 – 0M) in 0.05 M Tris, pH 7.0, and fractions (2.0 mL each) were monitored at 280 nm. The hemagglutinating activity was detected in the presence of 3% rabbit trypsin-treated erythrocytes (B) Size exclusion chromatography on Sephacryl S300 profile. Approximately 5 mg of proteins retained on Phenyl-Sepharose were applied to the column equilibrated with TBS/Ca^{2+}, pH 7.6, and fractions (3.0 mL each) were monitored at 280 nm. The hemagglutinating activity was detected in the presence of 3% trypsin-treated rabbit erythrocytes.

of acetone precipitation, hydrophobic interaction chromatography (Fig. 2A) and size-exclusion chromatography (Fig. 2B). Compared to the activity in crude extract, the specific lectin activity was increased 21 times by purification procedure. Purified CvL-2 represented 4.5 % of the total HA of the extract (Table III). This procedure also resulted in the copurification of another lectin from *C. varians*: CvL-1 (Fig. 2B). CvL-1 was purified 60 times from the crude extract and represented 48 % of total HA of the extract (Table III).

Molecular Mass Estimation of the Purified Lectins

The apparent molecular mass of the purified native HGL was estimated by gel filtration on calibrated TSKgel G3000SWXL column. It was calculated as 17 kDa (data not shown). In addition, on SDS-PAGE, under reducing and nonreducing conditions, HGL showed an apparent molecular mass of 15 kDa (Fig. 3A). These results suggest that HGL is a monomeric lectin, devoid of intermolecular disulfide bonds. On the other hand, native CvL-2 is a 465 kDA protein by native PAGE (Fig. 3B). On size-exclusion, CvL-2 was eluted in void-volume, suggesting a molecular mass higher than 1,000 kDa (data not shown). SDS PAGE of purified lectin showed a single band of 175 kDa, under reducing and nonreducing conditions (Fig. 3C). These data suggest that CvL-2 presents anomalous behavior. While appears as a trimeric protein on N-PAGE, it shows multimeric form on size-exclusion chromatography.

TABLE III
Purification processes of CvL-2. MAC – Minimum agglutinating concentration.

Fractions	Total protein (mg)	Titer	H.U./mg	Total activity	Purification	Yield (%)	MAC (µg/mL)
Crude Extract	166	256	154.2	25600	1	100	6.48
CvL-enriched fraction	19.35	512	1190.7	23040	7.7	90	0.84
Phenyl-Sepharose	1.76	1024	12800	22528	83	88	0.08
Sephacryl S-300 (CvL-2)	0.36	128	3200	1152	20.7	4.5	0.31
Sephacryl S-300 II (CvL-1)	1.32	1024	9309.1	12288	60.3	48	0.11

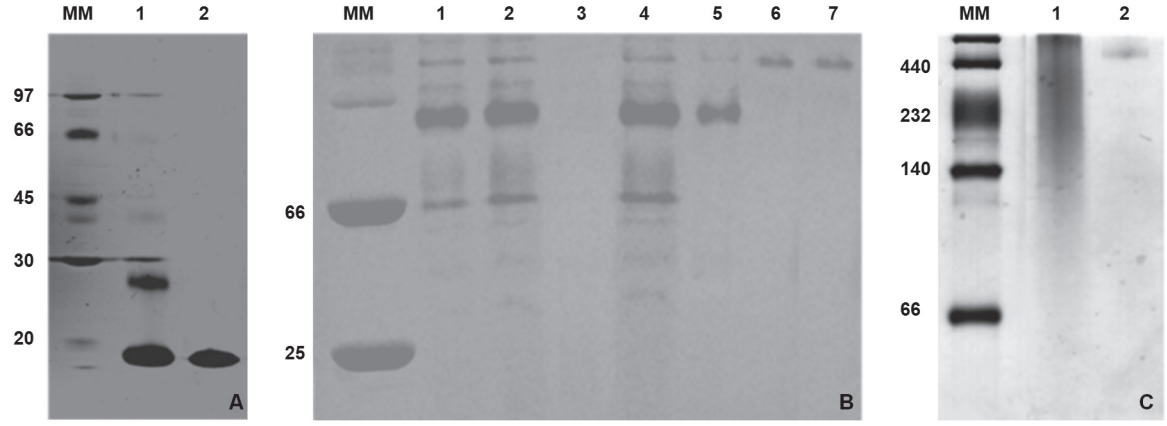

Figure 3 - Electrophoresis profile of purified lectins. (A) SDS-PAGE 15 % analysis of (M) Molecular Weight Markers: 1) *H. grisea* crude extract; 2) Fraction 2; 3) HGL in the presence of β-mercaptoethanol. (B) SDS-PAGE 10 % analysis of (M) Molecular Weight Markers: 1) *C. varians* crude extract; 2) CvL-enriched fraction; 3) precipitated proteins with $(NH_4)_2SO_4$ 1M; 4) Retained fractions on Phenyl Sepharose; 5) CvL-2; 6) CvL-2 in the presence of β-mercaptoethanol. (C) Native PAGE (7.5% acrylamide) analysis of (M) Molecular Weight Markers: 1) *C. varians* crude extract and 2) CvL-2. Proteins were stained with Coomassie blue and then with silver.

Hemagglutinating and Inhibition Assays of Purified Lectins

HGL quickly hemolyzed all tested erythrocytes. However, at the beginning of the hemolysis process, it was possible to observe the agglutination of erythrocytes. We were unsuccessful in achieving the inhibition of hemolytic activity with sugars, therefore, we conducted an osmotic protection assay. With this procedure, it was possible to observe that the dextrose was able to protect against erythrocyte lysis at the last dilution of HGL. This allowed us to visualize the agglutination by optical microscopy (Fig. 4) and perform the inhibition test. Hemagglutinating activity of HGL was inhibited by PSM and fetuin with minimal inhibitory concentration (MIC) at 0.31 mg/mL and 2.5 mg/mL, respectively (Table IV).

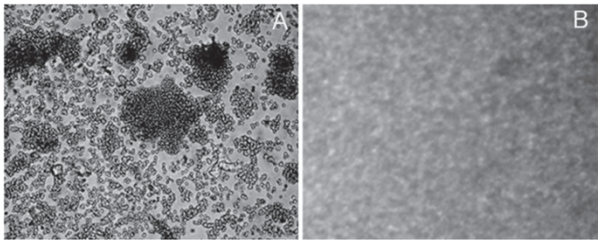

Figure 4 - Hemagglutinating activity of HGL under osmotic protection with 5.5% dextrose. **A)** Hemagglutinating of rabbit erythrocytes treated with trypsin in the presence of HGL; **B)** Control group: erythrocytes in saline.

CvL-2 strongly agglutinates trypsin-treated rabbit erythrocytes and, to a lesser degree, trypsin-treated human erythrocytes derived from blood type A. Hemagglutinating activity of CvL-2 was inhibited by galactosides and porcine stomach mucin (PSM). GalNAc and PSM were potent inhibitors of CvL-2 activity with MIC at 0.2 mM and 9.7 µg/mL, respectively. Galactose (MIC=12.5 mM), α-methyl-D-galactopyranoside (MIC=6.25 mM) and lactose (MIC=3.1 mM) were all able to inhibit CvL-2 (Table IV).

TABLE IV
Inhibition of hemagglutinating activity of the purified lectins. The initial concentrations were 100 mM and 5 mg/mL for sugars and glycoprotein, respectively.

Sugars	CvL-2 (MIC*)	HGL (MIC*)
D-Galactose	12.5 mM	NI
D-Glucose	NI	NI
D-Mannose	NI	NI
D-Arabinose	NI	NI
D-Frutose	NI	NI
Methyl-α-D-Galactopiranoside	6.25 mM	NI
Methyl-α-D-Glucopiranoside	NI	NI
D-GalNAc	0.2 mM	NI
D-GlcNAc	NI	NI
D-Lactulose	NI	NI
D-Sucrose	NI	NI
α-D-Lactose	3.1 mM	NI
Glycoproteins		
PSM	9.7 $\mu g.ml^{-1}$	0.31 $mg.ml^{-1}$
Tyroglobulin	NI	NI
Ovomucoid	NI	NI
Fetuin	NI	2.5 $mg.ml^{-1}$
Orosomucoid	NI	NI

Effects of pH, Temperature and Divalent Cations

Hemolytic activity of HGL was not inhibited when previously treated with EDTA. Although we added the divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+}, no change in protein activity was observed. HGL hemolytic activity was stable in all ranges of tested pH (4-10). HGL exhibited high thermostability. The hemolytic activity was unaffected after exposure of the lectin at 90 °C, and after 1h at 100 °C, the activity decreased to 50%.

Hemagglutinating activity of CvL-2 was completely abolished after dialysis against EDTA. The activity of the lectin was restored when EDTA was removed and 5 mM $CaCl_2$ was added to the protein solution. The lectin activity of CvL-2 was optimum on neutral pH (7). The activity rapidly decreased when the lectin was incubated in basic or acidic pH. CvL-2 showed relative thermostability. Its activity was stable at 50 °C, but gradually decreased with increasing temperature. At 90 °C, the activity remained at 25%, but it was completely abolished at 100 °C.

Toxic Effects of Crude Extracts and Lectins Purified from *C. varians* and *H. grisea*

Extracts from *C. varians* and *H. grisea* showed different levels of toxicity on *Artemia* nauplii. Crude extract of *H. grisea* was very toxic (LC_{50}= 8.1±3.0 µg/mL), whereas crude extract of *C. varians* showed low toxicity (LC_{50}= 116±23.3 µg/mL). The toxic effects were observed to be dose-dependent. The lectins HGL and CvL-1 showed potent activity against *Artemia* nauplii. LC_{50} values obtained were 9.5 µg/mL and 5.6 µg/mL, respectively. However, CvL-2 showed no toxicity against *Artemia* nauplii (LC_{50} = 850.1±3.4 µg/mL).

DISCUSSION

In at least twelve tested species, hemagglutinating activity was different for different blood types, suggesting the presence of lectin-like molecules in these species; namely, hemagglutinating activity was observed in 60% of the tested species. This value is similar to those found in other screenings of hemagglutinating/hemolytic activity of marine invertebrates (Dresch et al. 2005, Mojica et al. 2005). Furthermore, while hemolytic molecules, for the most part remain unknown, hemolytic activity was found in the extracts of some studied species, and HGL; which seems to be a hemolytic agent, was isolated in *Holothurea grisea*.

Toxic activity is a common factor among lectins (Pajic et al. 2002, Queiroz et al. 2008, Santos et al. 2010). Similar to lectins isolated from *Haliclona caerulea* (Carneiro et al. 2013), *Axinella corrugata* (Dresch et al. 2011) and *Aplysia kurodai* (Kawsar et al. 2010), HGL showed a high level of cytotoxicity against *Artemia* nauplii. However, hemolysis caused by lectin is rare, and few lectins have shown hemolytic activity (Hatakeyama et al. 1994, Sudhakar and Vincent 2014). The lectin isolated from the sea cucumber *Cucumaria echinata* (CEL-III) showed both hemolytic and toxic activities (Hatakeyama et al. 1994, Oda et al. 1997). CEL-III is an R-type lectin that is Ca^{2+}-dependent and gal/galNAc-binding (Nakano et al. 1999). These activities are mediated by binding of the protein to the specific carbohydrate chain on the target cells, followed by the formation of ion-permeable pores in the cell membrane through protein oligomerization (Hatakeyma et al. 1995). We were not able to determine the mechanism which mediates HGL-initiated hemolysis. However, both the agglutination of erythrocytes after osmotic protection and the HGL inhibition by PSM, indicated that the binding of HGL to carbohydrates in the erythrocyte membranes is the first step in hemolytic activity.

HGL showed singular stability, maintaining its activity in a broad range of pH and temperature. Usually, lectins from marine invertebrates possess optimal activity on basic-neutral pH and thermal stability up to temperatures of 70 °C.

Lectins isolated from sea cucumbers are generally ion-dependent proteins, many of which sharing structural factors that allow them to be grouped into the C-type super family (Himeshima et al. 1994, Hatakeyama et al. 2002), but HGL could not be grouped into the C-type super family . In general, C-type lectins bind to monosaccharides, such as mannose and galactose. HGL is not an ion-dependent lectin and does not bind to galactose or mannose. On the other hand, CvL-2 could be inhibited by EDTA, indicating that CvL-2 activity was Ca^{2+}-dependent. Lectins isolated from the sponges *Aplysina lawnosa*, *Aplysina archeri* and *Aphrocallistes vastus* also presented Ca^{2+}-dependent activity (Miarrons and Fresno 2000, Gundacker et al. 2001). However, it is impossible to define CvL-2 as a C-type lectin without knowledge of its primary structure.

Like CvL-2, a considerable number of lectins isolated from marine sponges, agglutinate protease-treated rabbit erythrocytes (Miarrons and Fresno 2000, Pajic et al. 2002, Dresch et al. 2008). CvL-2 was inhibited by diverse galactosides and PSM. Similar to CvL-1, CvL-2 was inhibited by galactose, but to a lesser degree (Moura et al. 2006). Moreover,

CvL-2 was inhibited by lactose at MIC=3.1 mM, whereas CvL-1 showed MIC=25 mM to some disaccharides (Moura et al. 2006). CvL-1 showed a slight affinity to glucose and its derivatives (Moura et al. 2006), but CvL-2 did not. The inhibition test for both lectins suggests that C-4 hydroxyl represents a point of differential recognition for CvL-1 and CvL-2. For CvL-2, C-4 hydroxyl likely interacts directly with CRD, participating in recognition, since galactose, but not glucose, was able to inhibit the activity of CvL-2. GalNAc was the powerful inhibitor for CvL-2 activity, most likely because the presence of *N*-Acetyl group increases the number of interactions between CvL-2 CRD and sugar. In fact, several lectins from marine invertebrates have presented affinity to GalNAc (Belogortseva et al. 1998, Beisel et al. 1999).

Molecular mass of CvL-2 in electrophoresis suggests a trimeric conformation (465 kDa). Several quaternary arrangements have been reported in sponge lectins: dimeric (Pajic et al. 2002), trimeric (Xiong et al. 2006), tetrameric (Miarrons and Fresno 2000) and multimeric (Medeiros et al. 2010) proteins linked by weak interactions or disulfide bonds were described.

Furthermore, similar to other lectins from marine invertebrates (Moura et al. 2006, Dresch et al. 2008), optimal activity of CvL-2 was observed in neutral pH, probably because that pH represents the natural environment of sponges.

Many extracts of aquatic organisms have been tested against *Artemia* nauplii and showed toxic activities (Thompson et al. 1985, Carbalho et al. 2002). Some of these compounds have already been isolated and evaluated in other models. The results have shown a range of biological activities, such as anticancer, suppression of the HIV virus, anti-inflammatory and proinflammatory activity (Herencia et al. 1998, McCune et al. 1989). In a previous study, CvL-1 was able to inhibit the growth of human leukemia cells, triggering the signaling of programmed cell death (Queiroz et al. 2008). CvL-1 also showed a high level of toxicity against *Artemia* nauplii. The new lectins isolated in this work showed different levels of toxicity to *Artemia* nauplii. HGL was highly toxic and could, like CvL-1, be a powerful biotechnology tool in cancer research. On the other hand, CvL-2 showed low toxicity effects and is expected to have no effect on malignant cells and inflammatory response.

In summary, hemagglutinating activity was observed in about 60% of tested species, indicating that marine invertebrates from the Brazilian coast are a rich and untapped source of new lectins. Furthermore, two new lectins were isolated, and one, HGL, is a rare lectin with hemolytic activity. Thus, our cataloging of lectin activity on marine invertebrates leads us to novel features associated with these marine organisms from a biotechnological perspective.

ACKNOWLEDGMENTS

This work was supported by the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) and Financiadora de Estudos e Projetos (FINEP). The authors are grateful to Msc. Sula Salani for the identification of the sponges. AHS, BSC, CSN adn KSN are senior investigators of CNPq.

RESUMO

Vinte espécies de invertebrados marinhos, coletados na costa brasileira, foram avaliados quanto à presença de atividade hemaglutinante/hemolítica. Em pelo menos doze espécies testadas, a atividade hemaglutinante foi diferencial para diferentes grupos sanguíneos, sugerindo a presença de lectinas. Extratos de quatro espécies apresentaram atividade hemolítica. Duas novas lectinas foram purificadas da esponja marinha *Cliona varians* (CvL-2) e do pepino-domar *Holothuria grisea* (HGL). CvL-2 foi capaz de aglutinar eritrócitos de coelho e foi inibida por galactosídeos. A atividade hemaglutinante foi estável em pH neutro e temperaturas abaixo de 70 °C. CvL-2 é uma

proteína trímero com subunidades de 175 kDa. Por outro lado, HGL apresentou atividade hemaglutinante e hemolítica em eritrócitos humanos e de coelho, mas a hemólise pôde ser inibida por proteção osmótica, enquanto que a aglutinação foi inibida por mucina. HGL mostrou-se estável em valores de pH de 4-10 e temperaturas até 90 °C. Em eletroforeses e gel filtração, HGL foi uma proteína monômero de 15 kDa. CvL-2 e HGL mostraram diferentes níveis de toxicidade contra náuplios de *Artemia*. CvL-2 apresentou LC_{50} de 850,1 µg/mL, enquanto que HGL apresentou LC_{50} de 9,5 µg/mL.

Palavras-chaves: purificação, hemólise, citotoxicidade, galactose.

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