An Acad Bras Cienc (2023) 95(Suppl. 2): e20220619 DOI 10.1590/0001-3765202320220619
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Purification, biochemical characterization of a lectin from marine sponge *Ircinia strobilina* and its effect on the inhibition of bacterial biofilms

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Abstract: A new lectin from marine sponge *Ircinia strobilina*, denominated IsL, was isolated by combination of affinity chromatography in Guar gum matrix followed by size exclusion chromatography. IsL was able to agglutinate native and enzymatically treated rabbit erythrocytes, being inhibited by galactosides, such as α-methyl-D-galactopyranoside, β-methyl-D-galactopyranoside and α-lactose. IsL hemagglutinating activity was stable at neutral to alkaline pH, however the lectin loses its activity at 40° C. The molecular mass determinated by mass spectrometry was 13.655 ± 5 Da. Approximately 40% of the primary structure of IsL was determined by mass spectrometry, but no similarity was observed with any protein. The secondary structure of IsL consists of 28% α-helix, 26% β-sheet, and 46% random region, as determined by dichroism circular. IsL was a calcium-dependent lectin, but no significant variations were observed by circular dichroism when IsL was incubated in presence of calcium and EDTA. IsL was not toxic against Artemia nauplii and did not have antimicrobial activity against bacterial cells. However, the IsL was able to significantly inhibit the biofilm formation of *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Key words: marine sponge, lectin, antibiofilm effect, amino acid sequencing.

INTRODUCTION

Lectins are ubiquitous proteins or glycoproteins that recognize and interact with specific carbohydrates in a reversible way, without, however, altering the structures of their ligands. These proteins have been identified and isolated from several organisms, including viruses, bacteria, algae, plants and animals (Sharon & Lis 2004).

In animals, lectins have been associated to both immunological and non-immunological functions (Yongqing et al. 2012). Lectins play an important role in defense mechanisms, recognizing invaders, damaging independent cells and mediating the biological activity of cells (Wang et al. 2019, Dang et al. 2018). In addition to their involvement in self/non-self recognition, lectins have been associated with several physiological functions, including participation in cell aggregation, fertilization, embryonic development, symbiosis, metamorphosis, and transport of complex sugars (Gundacker et al. 2001, Springer et al. 2008, Motohashi et al. 2017, Kilpatrick 2002, Zhou et al. 2017).

In sponges, lectins have a similar function to antibodies due to their ability to bind to bacteria or exogenous cells (Schroder et al. 2003, Funayama et al. 2005, Garderes et al. 2015). This

role is quite important, since invertebrates do not have an adaptive immune system (Smith et al. 1999). However, other biological functions are also attributed to sponge lectins suggesting that these proteins could be involved in symbiosis, cellular interactions and morphogenesis, cell aggregation, spicule formation, growth regulation and oxidative photoprotection (Gundacker et al. 2001, Carneiro et al. 2015, Garderes et al. 2015, Müller et al. 1981, Funayama et al. 2005).

In the biotechnological field, sponge lectins showed a high potential, having already been reported several effects caused by these molecules, *in vivo* and *in vitro*. For example, CchGs from *Cinachyrella* sp. was able to function as mammalian ionotropic glutamate receptors (Ueda et al. 2013); CCL from *Chondrilla caribensis* showed leshimanicide effect (Sousa et al. 2021); lectins from *Cinachyrella appion* and *Haliclona caerulea* showed pro apoptotic effect against human cervical adenocarcinoma cells and human breast cancer cells, respectively (Rabelo et al. 2012, Nascimento-Neto et al. 2018). Moreover, lectins from *Aplysna lactuca* and *A. fulva* were able to reduce bacterial biofilms (Carneiro et al. 2019, 2017a).

Till this date, there are no reports of lectins isolated of marine sponges from the *Ircinia* genus. Mebs and coworkers (1985) described the hemagglutinating activity and the toxicity in aqueous extracts of *Ircinia strobilina*, but no lectin was isolated so far (Mebs et al. 1985). In this work we isolated and characterized a new lectin from *Ircinia strobilina* (IsL). Moreover, the toxicity on *Artemia* nauplii, and antibacterial activity of IsL was evaluated.

MATERIALS AND METHODS

Reagents

Salts and buffers, including Tris(hydroxymethyl) aminomethane, sodium chloride, calcium chloride, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, glycine, dithiothreitol (DTT), iodoacetamide (IAA), etc.) were purchased from Sigma Aldrich. All carbohydrates, glycoproteins, standard proteins, enzymes and molecular weight kits were purchased from Sigma Aldrich. Mass spectrometry grade reagents, including acetonitrile, were purchased from Tedia. Artemia cysts were purchased from local store. Tryptone soy agar (TSA) medium, tryptone soy broth (TSB) and crystal violet were purchased from Merck Brazil.

Animal collection

Specimens of the *I. strobilina* were collected in tide pools of Icaraí beach, Amontada, state of Ceará, Brazil. After collection, sponges were cleaned from sand and epibionts and stored at -20°C. All collections were authorized by environmental organs under our inscription on SISBIO (Biodiversity Authorization and Information System, ID: 33913-8).

The species was identified, and a voucher was deposited (ID: UFPEPOR 2693) at the Zoology Department of the Universidade Federal de Pernambuco, PE, Brazil.

Purification of IsL

The access to the genetic heritage of *Ircinia strobilina* was authorized by the competent institutions through our registration in SISGEN (National System for the Management of Genetic Heritage and Associated Traditional Knowledge, ID: A1792FE).

Sponges were cut in to small pieces, homogenized with the 25 mM tris buffer, pH 7.6, containing 0.15 M NaCl and 20 mM $CaCl_2$ (TBS / $CaCl_2$), in the proportion of 1:3 (w/v). The mixture was centrifuged at 5000 x *g* for 20 minutes at 4 °C. The obtained supernatant (crude extract) was subjected to determination of total soluble

proteins (Bradford 1976) and hemagglutination assays (Sampaio et al. 1998).

Then, the crude extract was centrifuged and subjected to affinity chromatography on a Guar gum column (4.5 x 2.5 cm), previously equilibrated with the extraction buffer (TBS/$CaCl_2$). A flow rate of 1 mL.min^{-1} was maintained and absorbance was monitored at 280 nm in an Ultrospec 2100 pro spectrophotometer (Amershan). The non-retained proteins in the affinity matrix were washed with the equilibration buffer until the effluents showed absorbance values below 0.02. Proteins adsorbed were eluted with 25 mM Tris buffer, pH 7.6, containing 0.15 M NaCl and 20 mM EDTA (TBS/EDTA). Two-mL fractions were collected and those that showed hemagglutinating activity were pooled, dialyzed against distilled water, lyophilized and used for size exclusion chromatography (SEC).

For SEC, a Biosuite ™ 250 HR SEC column (0.78 x 30cm, particle size 5 µm) was coupled to an Acquity UPLC ™ system (Waters Corp.). The column was equilibrated and eluted with TBS/$CaCl_2$ buffer pH 7.6 at a flow rate of 0.5 mL.min^{-1}, 0.5 mL-fractions were collected and submitted to hemagglutinanting assay. The lectin fractions (*Ircinia strobilina* lectin, IsL) were combined, dialyzed against distilled water and lyophilized.

Hemagglutination and hemagglutination inhibition assays

The hemagglutination assay was performed in microtiter plates with V-bottom wells using the twofold serial dilution method (Sampaio et al. 1998). Human ABO and rabbit erythrocytes were used in both forms: native and treated with proteolytic enzymes (papain and trypsin). A hemagglutination unit (H.U) was defined as the inverse of the highest dilution of a given solution that is still capable of agglutinating a 3% suspension of erythrocytes after one hour.

The hemagglutinating activity inhibition assay was performed according to the method described by Sampaio et al. (Sampaio et al. 1998), using the following sugars and glycoproteins: α-methyl-D-galactopyranoside, β-methyl-D-galactopyranoside, 4-nitro- phenyl-α-D-galactoside, nitrophenyl-β-galactoside, methyl-galactoside, O-nitro-phenyl-β-D-galactoside, nitrophenyl-β-galactoside, α-lactose (Galβ1-4α-Glu), melibiosis (Galα1 -6Glu), fucose, phenyl-β-D-thiogalactopyranoside, L-rhamnose, N-acetyl-galactosamine, ribose, L-arabinose, D-galactose, D-mannose, lactulose, glucosamine, D-glucose, Ralphose, fructose, sucrose, GlcNac, xylose, maltose, type II and type III porcine stomach mucin.

SDS-PAGE and molecular mass determination

SDS-PAGE in the presence and absence of β-mercaptoethanol, stained with Coomassie (Laemmli 1970) was used to estimate homogeneity of IsL under denaturation conditions. LMW-SDS marker kit (GE Healthcare, UK) was used as a standard.

The molecular mass of the native lectin was estimated using size-exclusion chromatography on Biosuite ™ 250 HR SEC column (0.78 x 30cm, particle size 5 µm) coupled to an Acquity UPLC ™ system (Waters Corp.), at a flow rate of 0.4 mL.min-1. The column was equilibrated and eluted with TBS/$CaCl_2$ pH 7.6, and the molecular mass of the lectin was estimated according to the comparison of its elution time with the following standards: BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and lysozyme (14 kDa).

The average molecular mass of IsL was determined through mass spectrometry using the MALDI ionization source. The instrument (Autoflex MALDI-TOF, Bruker-Daltonics) operated with a voltage of 20 kV in linear mode and in the range of mass/load ratio (*m/z*) from 20,000

to 80,000. The matrix solution used was alpha-cyano-4-hydroxycinnamic acid (10 mg.mL^{-1} of CHCA on acetonitrile, water, trifluoroacetic acid, 50:47:3% v/v). The acquired spectra were processed with FlexAnalysis software.

Effects of pH, temperature, EDTA, divalent ions and sugar content

The effects of pH, temperature, EDTA and divalent ions on lectin activity were evaluated as described by (Sampaio et al. 1998). The content of neutral carbohydrates in IsL was evaluated, as described by Dubois et al. (Dubois et al. 1956), using lactose as a standard.

Tandem Mass spectrometry analysis of IsL

Digestion with trypsin was performed in 50 mM ammonium bicarbonate. Firstly, an electrophoresis was conducted as described above. After coloration, protein spots were excised, reduced and carboxyamidomethylated as described by Shevchenko et al., (Shevchenko et al. 2006). Digestion was maintained at 37°C for 16 h, when then it was stopped by addition of 2% formic acid.

Peptides generated by tryptic digestion were extracted as described by (Shevchenko et al. 2006). Peptide solutions were loaded on a C-18 nano column (0.075 x 100 mm) coupled to a nanoACQUITY system (Waters Corp). The chromatography eluates were infused into a nanoelectrospray source of a hybrid mass spectrometer (Synapt HDMS system-Waters Corp).

The mass spectrometer operated in a positive mode, with the source temperature of 373 K, under capillary voltage of 3.0 kV and calibrated with the fragmentation pattern of [Glu1] -fibrinopeptide B. Data collection was performed according to the data dependent analysis (DDA) method. The reference used during data collection was the 785.84 *m/z* ion of [Glu1]-fibrinopeptide B. The selected ions were fragmented by CID (Collision Induced Dissociation), using argon as the fragmentation gas. All CID spectra were interpreted manually and searches for similarity were carried out online using BLAST on the NCBI website.

Circular Dichroism

Circular Dichroism (CD) measurement was performed according to Carneiro et al. (2017a) using a Jasco J-815 spectropolarimeter (Jasco International Co., Tokyo, Japan) connected to a peltier with controlled temperature. IsL was evaluated under two distinct conditions: (1) in presence of Ca^{2+} (lectin solubilized in TBS/Ca); and (2) in absence of Ca^{2+} (lectin solubilized in TBS/EDTA).

The secondary structure quantification was estimated by Dichroweb (Whitmore & Wallace 2004), using the CONTIN algorithm (Sreerama & Woody 2000).

Artemia lethality test

The Artemia sp. nauplii Lethality test was performed as described by Carneiro et al. (Carneiro et al. 2013b). The cysts were incubated with artificial sea water (1g of cysts/L of sea water), under constant lighting and aeration at 28°C. After 48 hours, aeration was suspended and the nauplii were collected for testing.

Lectin in different concentrations (12.5; 25; 50; 100 µg.mL^{-1}) was added to 24-well Linbro plates (Hampton Research 34 Journey Aliso Viejo, CA USA), in which each well contained 10 Artemis nauplii in one final volume of 2 mL. The assay was performed in triplicate, and the negative control consisted of wells that contained 2 ml of artificial seawater with 10 Artemia nauplii. After 24 h, the number of nauplii killed in each well was counted. The percentage of deaths at each concentration was calculated and the LC50

value was determined using a Probit analysis, as described by Finney (Finney 1952).

Evaluation of antibacterial and antibiofilm activity

Bacterial strains and culture conditions

Staphylococcus aureus ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, and *Escherichia coli* ATCC 11303 were used in this study, all obtained from the American Type Culture Collection (ATCC). The bacteria were grown in a Tryptone Soy Agar (TSA) medium and incubated at 37°C for 24 h. Colonies isolated were inoculated into 5 mL of Tryptone Soy Broth (TSB) for 18 h at 37°C. After growth, the bacterial suspension was adjusted to 1×10^6 colony-forming units ($CFU.mL^{-1}$) through turbidimetry (620 nm) with calibration curves previously determined for each bacterium.

Effects of IsL on planktonic cells

The effect of IsL on planktonic cultures was determined by the broth microdilution test, according to the Clinical and Laboratory Standards Institute (CLSI), with modifications (Vasconcelos et al. 2014, Carneiro et al. 2017a). Lectin was tested in concentrations ranging from 3.9 to 250 $\mu g.mL^{-1}$.

Biofilm formation assay

The biofilm formation test was evaluated according to Stepanovic et al (Stepanović et al. 2000), with modifications (Carneiro et al. 2017a, Vasconcelos et al. 2014). The assay was performed by adding 100 µL of each bacterium (1×10^6 CFU mL^{-1}) with 100 µL of different concentrations of lectin (3.9 to 250 $\mu g.mL^{-1}$). The plates were incubated at 37°C for 24 h for the development of biofilms. After the biofilms development, the content of each well was removed and the biofilms were subjected to two washes with 200 µL/well of sterile water, in order to remove weakly adherent cells. The entire procedure was performed on two separate microtiter plates, one for the total biomass quantification, and a one second plate for the number of viable cells present enumeration.

To quantify the biofilm biomass, the colorimetric crystal violet (CV) method was used. In this test, 200 µL of 99% methanol was added to each well for 5 min . After the methanol solution was removed the plates were dried at 25 °C. To stain the biofilms formed, 200 µL of 1% CV was added to the wells and after 5 min the excess dye was removed; the plates were washed with sterile water and dried at 25 °C. Finally, 200 µL of 33% acetic acid solution was added to the wells to dissolve the violet crystal. Then, the optical density was measured at 590 nm (OD590) in a microtiter plate reader (SpectraMax®I3, Molecular Devices LLC, Sunnyvale, CA, USA).

Quantification of number of viable cells in the biofilms

To assess the viability of the biofilm-entrapped cells, the wells were washed twice with 200 µL of sterile water and the plates were placed in an ultrasonic bath operating at 50 kHz, for 8 minutes. From the bacterial suspensions obtained, decimal dilutions were prepared and plated in TSA. The plates were incubated for 24 h at 37°C, and the total number of colonies forming units (CFU) ($logCFU\ mL^{-1}$) were determined**.**

Statistical analysis

Statistical analysis were performed using the GraphPad Prism® version 7.0 for Microsoft Windows®. The data from all tests were compared using analysis of variance (ANOVA), with the Bonferroni post-hoc test. The data were considered significant when $p < 0.05$.

RESULTS

Purification of IsL

A lectin present in the marine sponge *Ircinia steobilina*, called IsL (*Aaptos* Lectin), was purified by combining affinity chromatography on Guar gum, followed by size exclusion chromatography. IsL was purified 117x and represented 0.5% of soluble proteins of *I. strobilina* (Table I).

Hemagglutination and hemagglutination inhibition assays

IsL was able to agglutinate only rabbit erythrocytes in both forms, native and treated with proteolytic enzymes (papain and trypsin). The hemagglutinating activity of IsL was inhibited by galactose derivatives and mucin (Table II).

SDS-PAGE and molecular mass determination

The apparent molecular mass of IsL was estimated at 21 kDa under reducing conditions and at 29 kDa, under non-reducing conditions (Figure 1). In the SEC, IsL showed a single symmetrical peak of native molecular mass estimated at 12 kDa (Figure 2).

The molecular mass of IsL, determined by MALDI-ToF mass spectrometry, was 13,655 ± 2 Da (Data not shown).

Effects of pH, temperature, EDTA, divalent ions and sugar content

The hemagglutinating activity of IsL was maximum at pH 7 (Figure 3a) and at temperatures below 40°C (Figure 3b). IsL was shown to be a calcium-dependent lectin, once its hemagglutinating activity was not observed in presence of EDTA.

IsL is not characterized as a glycoprotein, as determined by phenol-sulfuric acid method.

Tandem Mass spectrometry analysis of IsL

Six peptides of IsL were sequenced after digestion and mass spectrometry analysis (Table III). Peptides T3' and T4' showed microheterogeneities in comparison to T3 and T4, respectively. Together peptides T1, 2, 3 and 4 represented 57% of the IsL amino acid sequence.

However, no similarity was observed between IsL peptides and any proteins.

Circular Dichroism

IsL Far-UV spectra showed minimum absorption at 200 nm and maximum absorption at 250 nm (Figure 4a). The theoretical secondary structure of IsL lectin consists of 28% α-helix, 26% β-leaf, and 46% random region, according to the CONTIN forecast method, available online as part of the DICHROWEB server (http://dichroweb.cryst.bbk.ac.uk).

The presence of EDTA did not alter the IsL spectra (Figure 4b), suggesting that Ca^{2+} is not involved in the maintenance of IsL secondary structure.

Table I. Purification of IsL.

Fracion	Volume (mL)	Titer ($H.U.mL^{-1}$)	Protein total (mg)	H.U total	Specific activity ($H.U.mg^{-1}$)	Purification (times)	Yield (%)
Crude Extract	100	4	113.36	400	3.52	1	100
Guar gum	50	32	17.6	1,600	90.9	25.8	15
Size exclusion	24	256	0.576	6,144	10,667	117.3	0.5

H.U- Hemagglutinating unit.

Table II. Inhibition of the hemagglutinating activity of IsL by sugars and glycoprotein.

SUGARS	MIC*
α-methyl-D-galactopyranoside	5 mM
β-methyl-D-galactopyranoside	5 mM
4-nitrophenyl-α-D-galactoside	5 mM
O- nitrophenyl-β-D-galactoside	5 mM
α-lactose (galβ1◊4α-glu)	25 mM
Melibiosis (galα1◊6glu)	25 mM
Fucose	25 mM
Phenyl-β-D-thiogalactopyranoside	50 mM
L -rhamnose	50 mM
GalNAc	50 mM
Ribose	50 mM
Glycoproteins	
Porcine Stomach Mucin	0.031 mg.mL^{-1}

The following sugars did not cause inhibition: L-arabinose, D-galactose, D-mannose, lactulose, glucosamine, D-glucose, Ralphose, fructose, sucrose, GlcNac, xylose, maltose.
*** Minimum concentration of sugar required for inhibition.**

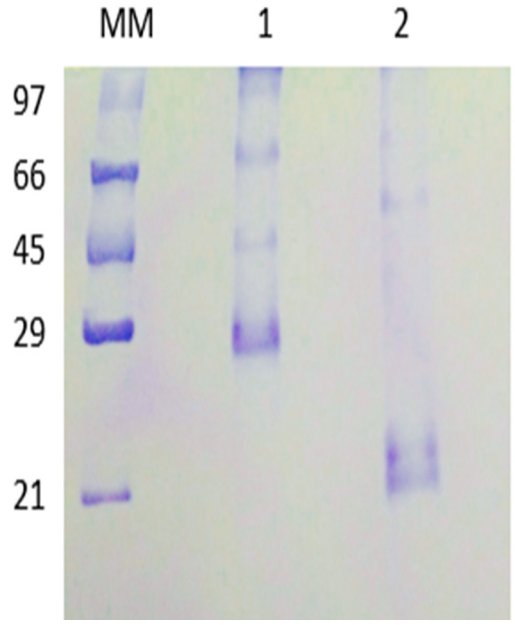

Figure 1. Electrophoresis in 12% polyacrylamide gel in the presence of SDS (SDS-PAGE). 20 µg of protein were applied to each well. Molecular marker MM (phosphorylase B 97,000 Da, BSA 66,000 Da, ovalbumin 45,000 Da, carbonic anhydrase 29,000 Da, ovomucoid 21,000 Da; non-reduced IsL (1), reduced IsL (2).

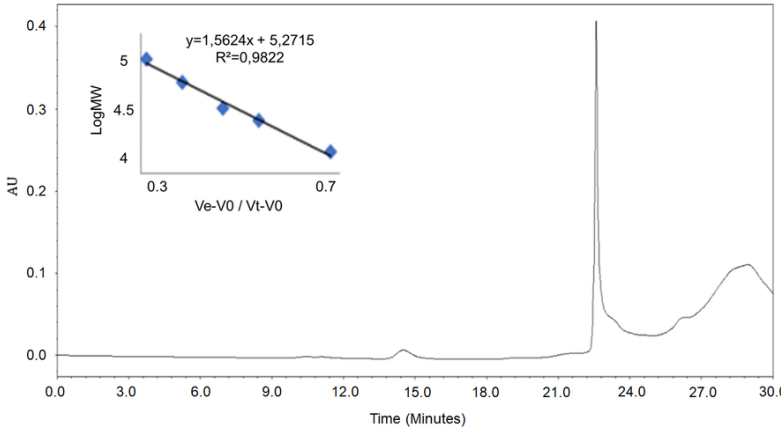

Figure 2. Estimation of native molecular mass by size exclusion chromatography. Size exclusion chromatography on a BioSuite 250 5µm HR SEC column (0.78 x 30 cm, flow: 0.4 mL/min), equilibrated with TBS/$CaCl_2$ buffer pH 7.6, applied volume of *IsL*: 500 µL. Calibration curve: (1) BSA, 66 kDa; (2) ovalbumin, 45 kDa; (3) carbonic anhydrase, 29 kDa; (4) trypsinogen, 24 kDa and (5) lysozyme, 14 kDa.

Artemia lethality test

The IsL did not show a toxic effect against Artemia sp nauplii by observing that all nauplii remained alive after inoculation with lectin in various concentrations.

Inhibition of bacterial biofilm formation

In the present study it was observed that IsL does not show antibacterial activity against planktonic cultures of the Gram-positive and Gram-negative bacteria tested. On the other hand, IsL was able to inhibit the biofilm formation of *Staphylococcus aureus* (Figure 5a and d) and *Staphylococcus epidermidis* (Figure 5b and e), significantly reducing the total biomass and number of viable cells of the biofilms of both bacterial strains. However, no effect of the lectin was observed on *E. coli* biofilm (Figure 5c and f).

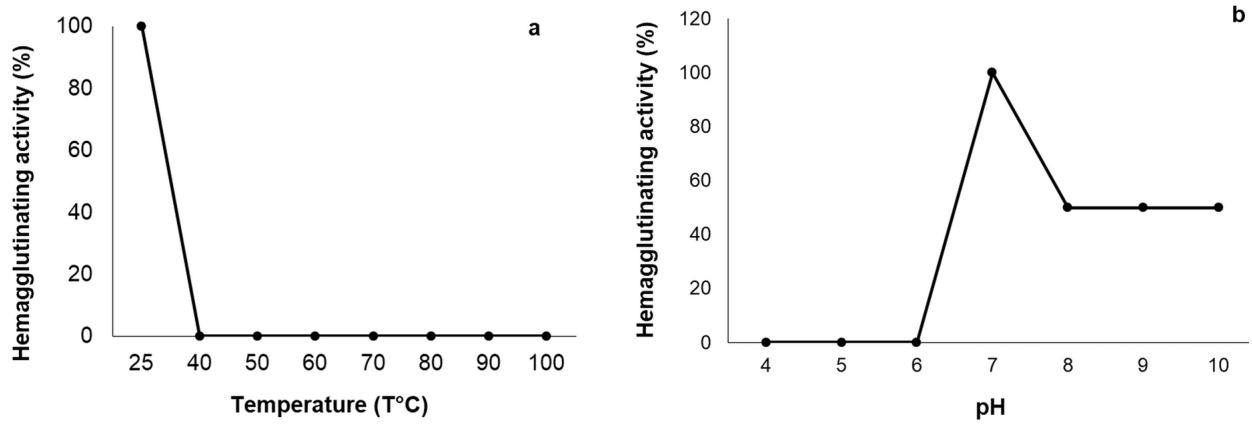

Figure 3. Physical Chemical properties of IsL Hemagglutinating activity. Effect of pH variation on hemagglutinating activity of lectin. Percentage of hemagglutination at different pH **(a)**. Effect of temperature variation on hemagglutinating activity of lectin. Percentage of hemagglutination at different temperatures **(b)**.

Table III. Amino acid sequence of peptides from the lectin of the marine sponge *Ircinia steobilina*.

Peptide	*m/z*	Amino acid sequence	Molecular mass		Δ (Da)
			observed	calculated	
T1	702.3490	SXXVXQQNWFR	1402.6824	1402.7721	0.09
T2	792.0416	XTDKWYSXVPSXGHDTXSWR	2373.1013	2373.2117	0.11
T3	766.7069	AXPPDGTVAXVXFQ-HDTYQGR	2297.0972	2297.1804	0.08
T3’	785.7440	AXPPDGTVAXVXFQGHDTYQGR	2354.2085	2354.2019	-0.01
T4	723.8245	AVFFEHTYYNR	1445.6334	1445.6727	0.04
T4’	480.8802	AVFYEHNQYNR	1439.6171	1439.6582	0.04

X Represents Leucine or Isoleucine.
’ Represents peptides wiht microheterogeneities in their sequences.

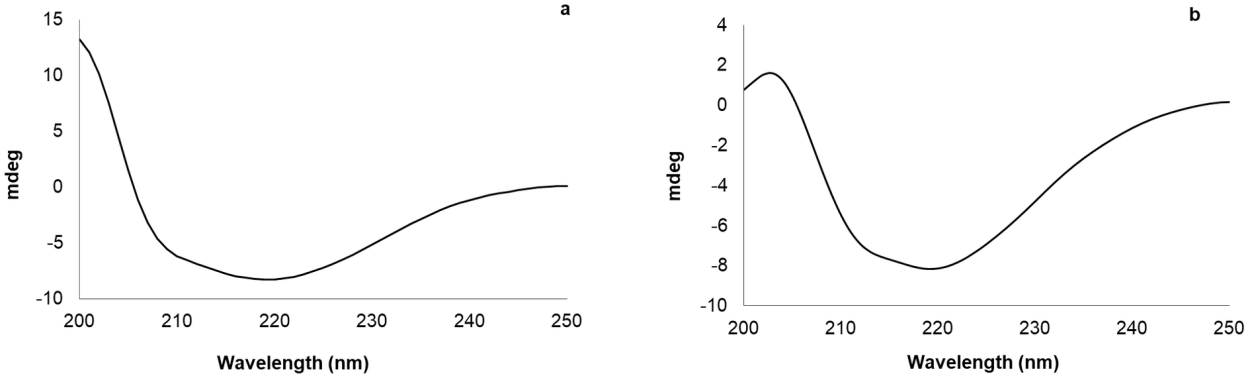

Figure 4. Far-UV CD spectra (200-250 nm) of IsL. The protein concentration was 200 µg.mL^{-1} in TBS/CaCl2 pH 7.6 (a). The protein concentration was 5 µg in TBS/EDTA pH 7.6 (b).

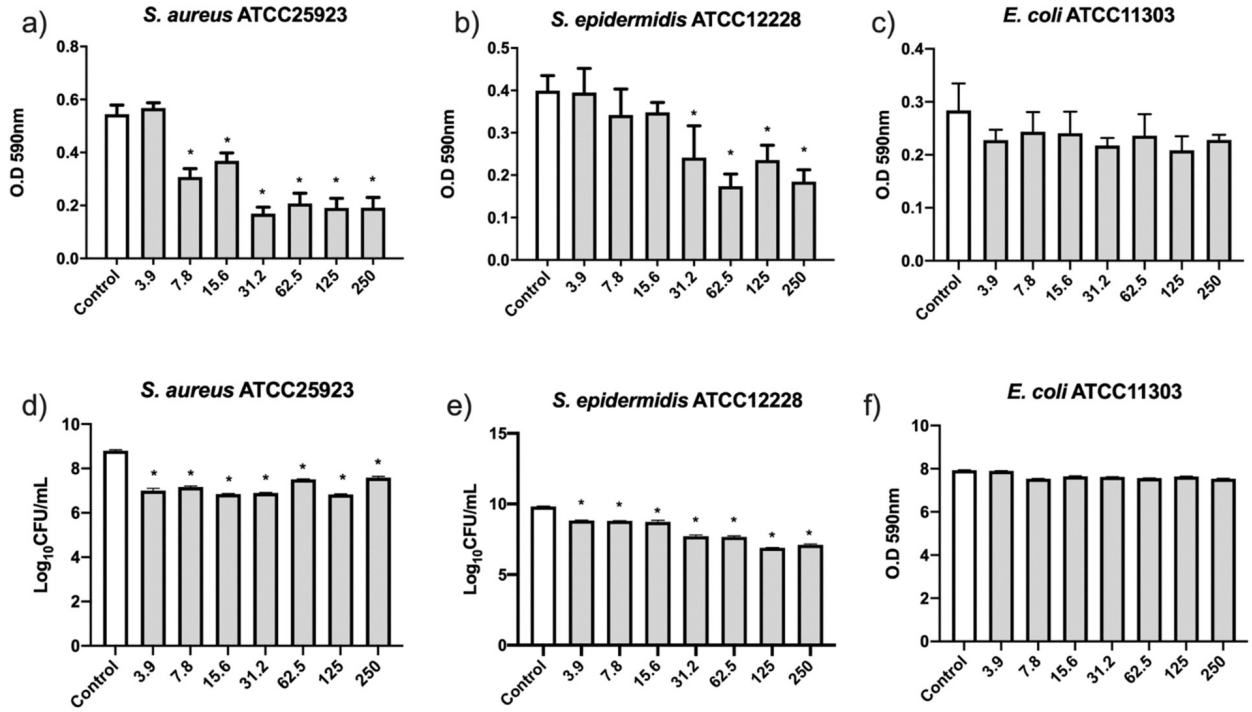

Figure 5. Biofilm formation of bacteria in the presence of different concentration of IsL. Quantification of total biomass by crystal violet staining for *S.aureus* (a), *S.epidermidis* (b) and *E.coli* (c). Mean values of the logarithm of colony forming units per mL for *S. aureus* (d), *S. epidermidis* (e) and *E.coli* (f) biofilms. * Significantly different ($p < 0.05$) compared to the control group. Error bars display standard deviation (SDs) of the means.

DISCUSSION

IsL was successfully purified by combination of affinity chromatography on Guar gum, followed by SEC. Other sponge lectins were also isolated using this same methodology (Medeiros et al. 2010, Dresch et al. 2008, Kawsar et al. 2008, Marques et al. 2018).

Inhibition assay results suggests that IsL is a galactophilic lectin, like most of the described sponge lectins (Garderes et al. 2015, 2019, 2017a, Kawsar et al. 2008, Ueda et al. 2013, Medeiros et al. 2010). IsL has also been inhibited by PSM, which is an *O*-linked glycoprotein containing galactose, GalNac and fucose residues (Devine & Harada 1991). Lectins from *Aplysina lactuca* (ALL) and *A. fulva* (AFL) were also inhibited by galactose containing-carbohydrates and glycoprotein PSM (Carneiro et al. 2019, Carneiro et al. 2017a). However, unlike IsL, ALL and AFL showed low affinity for simple carbohydrates.

Molecular mass of IsL observed in SDS PAGE was quite different (21 and 29 kDa, in reduced and non-reduced forms, respectively) that ones observed in SEC (13 kDa) and determined by MS (13,655 ± 2 Da). Together these data suggest that IsL is a dimmer formed by identical subunits linked by disulfide bonds, as observed in SDS PAGE, but monomer molecular mass is 13,655 ± 2 Da, which was observed in MS possibly like a double charged ion. Molecular mass estimated by SEC can be understood as an anomalous behavior of the lectin under the chromatography conditions.

Divergences between molecular mass determined by MS and mass estimated by SDS-PAGE are observed in some proteins (Ainouz et al. 1995, Carneiro et al. 2013a). These divergences can be attributed to intrinsic characteristics of

the protein, such as internal disulfide bonds, glycosylation, or phosphorylation (Carneiro et al. 2013a). Eventually, these characteristics modify the migration of a protein in electrophoresis, explaining the fact that IsL showed 21 kDa under reducing conditions and 29 kDa under non-reducing conditions.

Hemagglutinating activity of IsL was stable in neutral-alkaline buffers like other sponge lectins (Pajic et al. 2002, Moura et al. 2006, Carneiro et al. 2017a), but it was thermolabile, showing a rare condition of sponge lectins.

IsL has been shown to be a calcium-dependent lectin. This characteristic is common in marine invertebrate lectins (Moura et al. 2015, de Melo et al. 2014, Moura et al. 2013) and sponge lectins (Garderes et al. 2015, Moura et al. 2006).

Dependence on calcium was used as a determining factor in classifying lectin into C-type, but after several structural studies it became evident that C-type proteins have a characteristic carbohydrate recognition domain (CRD), formed by a double loop stabilized by two bridges of highly conserved sulfide located at the base of the second loop (Zelensky & Gready 2005). Thus, proteins containing a C-type lectin domain (CTLD - C-type lectin like) fit in the C-type. Therefore, to classify any lectin as type C, including IsL, several structural studies are necessary, since the calcium addition may be just a clue.

Tryptic peptides obtained from IsL showed no similarity with known proteins. The lack of similarity with other lectins does not allow the classification of IsL in the families of existing lectins, suggesting that the lectin isolated from *Ircinia steobilina* may become part of a new family of lectins.

Other sponge lectins also did not show amino acid sequence similarity with proteins deposited in databases, such as HMA - *Haliclona manglaris* agglutinin, and H-3 from *Haliclona caerulea* (Carneiro et al. 2015, 2013a).

IsL was able to reduce the biofilm formation of Gram-positive bacteria. In fact, the ability of lectins from marine organisms to inhibit the formation of bacterial biofilm has been reported in some studies, as observed in the lectins from the seaweed *Bryothamnion seaforthii* (BSL) and *Hypnea musciformis* (HML) that were able to inhibit the biofilm formation of the *S. aureus* (Vasconcelos et al. 2014). The lectin present in the eggs of *Aplysia dactylomela* (ADEL) inhibited the biofilm formation of *S. aureus*, especially at the highest concentrations tested (Carneiro et al. 2017b). Moreover, the lectin of the marine sponge *Aplysina lactuca* (ALL) was able to reduce the biomass and number of viable cells of both *S.aureus* and *E. coli* (Carneiro et al. 2017a).

Biofilms are complex structures and are more resistant to antibiotics than planktonic microorganisms. Biofilms are the cause of many chronic and persistent infections, so therapeutic treatments are more difficult and limited (Blackledge et al. 2013). Thus, it is important to find new molecules, such as lectins, that can act as inhibitors and/or to control the formation of bacterial biofilm (Vasconcelos et al. 2014).

In the present study, it was observed that IsL was able to inhibit the biofilm formation of *S. aureus* and *S. epidermidis*. The mechanisms by which lectins exert their antimicrobial activity are not well described, but it is possible that this effect occurs through interactions between the lectin and the bacterial cell wall components, including teichoic acids and teichuronic, peptidoglycans and lipopolysaccharides (Paiva et al. 2010). In this study, *E. coli* biofilm was not affected in the presence of the IsL, probably by the differential recognition of the lectin on carbohydrates present on the surface of Gram-positive and Gram-negative bacteria. Moura et al. (Moura et al. 2006) showed that the CvL, a

lectin isolated from the marine sponge *Cliona varians*, recognizes glycans on the bacterial surface and distinguishes Gram-positive from Gram-negative bacteria.

As stated earlier, lectins are important molecules, so further research is needed on their physiological functions, and on their potential as molecules/tools for new biotechnological and medical applications. Furthermore, the discovery of new sponge lectins is essential for studies to intensify and for more physiological and biological functions to be discovered.

Acknowledgments

This work was supported by the Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FUNCAP (Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico) and FINEP (Financiadora de Estudos e Projetos). M.A.V. acknowledges financial support from the CNPq (Grant number: 429670/2018-4) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Grant number: APQ-00224-21). The authors thank CETENE (Centro de Tecnologias Estratégicas do Nordeste) for access to the mass spectrometer. The authors are especially grateful to Dr. Julia Campos for MALDI-ToF analysis. The authors are grateful for the help in text editing. A.H.S., C.S.N., E.H.T and RFC are investigators of CNPq.

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How to cite

ALMEIDA AS, MENDONÇA DNM, CARNEIRO RF, PINHEIRO U, NASCIMENTO EF, ANDRADE AL, VASCONCELOS MA, TEIXEIRA EH, NAGANO CS & SAMPAIO AH. 2023. Purification, biochemical characterization of a lectin from marine sponge *Ircinia strobilina* and its effect on the inhibition of bacterial biofilms. An Acad Bras Cienc 95: e20220619. DOI 10.1590/0001-3765202320220619.

Manuscript received on July 25, 2022;
accepted for publication on February 9, 2023

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ASA developed methodologies for the isolation of the protein and wrote the draft of the article (Investigation; Writing - Original Draft). DNM performed chromatographies aiming at the accumulation of proteins for biological assays; RFC performed MS and CD experiments (Investigation). MAV performed the antibiofilm assays and revised the original draft (Investigation; Writing - Original Draft). ALA performed the antibiofilm assays; EFN identified the sponge (Investigation). UP validated the species and keep a voucher in his collection (Data Curation). EHT provided inputs and the laboratory to carry out the antibiofilm experiments; CSN provided inputs and the laboratory to carry out the mass spectrometry experiments (Resources). AHS provided inputs and laboratory for protein purification and characterization, revised the manuscript (Resources; Supervision). All authors read and approved the final manuscript.