

Purification and characterisation of a lectin from the red marine alga *Pterocladia capillacea* (S.G. Gmel.) Santel. & Hommers.

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ABSTRACT – (Purification and characterisation of a lectin from the red marine alga *Pterocladia capillacea* (S.G. Gmel.) Santel. & Hommers.). A lectin present in the marine red alga *Pterocladia capillacea* was purified and characterised by extraction of soluble proteins (crude extract) in 20 mM Tris-HCl buffer, pH 7.5. Among the analysed erythrocytes (human blood group A, B and O and the animals ox, goat, chicken and rabbit) the lectin agglutinated specifically rabbit erythrocytes. The hemagglutinating activity assay showed that the lectin was not dependent on divalent cations and was shown to be inhibited by the glycoproteins avidin and mucin. The purification procedure was conducted by precipitation of the crude extract with 80% saturation ammonium sulfate (F0/80) followed by affinity chromatography on guar-gum column. The lectin of *P. capillacea* was purified 14.5 fold and had a recovery of 27.4% of the original total specific activity present in the crude extract. The absence of carbohydrate suggested that the lectin is not a glycoprotein. The molecular mass of *P. capillacea* lectin, determined by gel filtration, was 5.8 kDa. SDS-PAGE in the presence of β -mercaptoethanol gave one band, indicating that the native lectin is a monomeric protein. The activation energy of denaturation process (ΔG°) was calculated to be 106.87 kJ . mol⁻¹ at 70 °C.

RESUMO – (Purificação e caracterização de uma lectina da alga marinha vermelha *Pterocladia capillacea* (S.G. Gmel.) Santel. & Hommers.). Uma lectina presente na alga marinha vermelha *Pterocladia capillacea* foi purificada e caracterizada pela extração de proteínas solúveis (extrato bruto) em tampão Tris-HCl 20 mM, pH 7,5. Entre os eritrócitos testados (grupos sanguíneos humanos A, B e O e os animais boi, cabra, galinha e coelho) a lectina aglutinou especificamente eritrócitos de coelho. O ensaio de atividade hemaglutinante evidenciou que a lectina não era dependente de cátions divalentes e inibida pelas glicoproteínas avidina e mucina. O procedimento de purificação foi conduzido por precipitação protéica do extrato bruto com sulfato de amônio até 80% de saturação (F 0/80), seguido por cromatografia de afinidade em coluna de goma de Guar. A lectina de *P. capillacea* foi purificada 14,5 vezes e teve uma recuperação de 27,4% da atividade específica total original presente no extrato bruto. A ausência de carboidrato sugeriu que a lectina não é uma glicoproteína. A massa molecular da lectina de *P. capillacea*, determinada por filtração em gel, foi de 5,8 kDa. PAGE-SDS em presença de β -mercaptoetanol mostrou uma única banda protéica, indicando que a lectina de *P. capillacea* é uma proteína monomérica. A energia de ativação do processo de desnaturação (ΔG°) foi calculada em 106,87 kJ . mol⁻¹ a 70 °C.

Key words - Agglutinin, algae, lectin, *Pterocladia capillacea*, purification

Introduction

Lectins constitute a group of proteins or glycoproteins of non-immune origin, which bind reversibly to carbohydrates and usually agglutinate cells or precipitate polysaccharides and glycoconjugates (Goldstein *et al.* 1980). The lectins were redefined by Peumans & Van Damme (1995) as proteins possessing at least one non-catalytic domain, which binds reversibly to a specific mono or oligosaccharide. However, according to Cummings (1997), antibodies and proteins with enzymatic activity related to

carbohydrates can not be considered as lectins. As a consequence of their chemical properties, they have become a useful tool in several fields of biological research (immunology, cell biology, membrane structure, cancer research and genetic engineering). Lectins are present in a wide range of organisms from bacteria to animals, being present in all classes and families, although not in all the kinds and species (Lis & Sharon 1981). The first report on the occurrence of lectins in marine algae is relatively recent (Boyd *et al.* 1966). Although several studies on lectins from marine algae have been reported, the number of these proteins purified and characterised is still considered small. These studies include the green algae *Codium tomentosus* (Huds.) Stackhouse (Fabregas *et al.* 1988), *Ulva lactuca* L. (Sampaio *et al.* 1998a) and *Caulerpa cupressoides* (Vahl) C. Agardh (Benevides *et al.* 2001);

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the brown algae *Fucus vesiculosus* L. (Ferreiro & Criado 1983), *Dictyota dichotoma* (Hudson) Lamouroux (Chiles & Bird 1989) and the red algae *Plumaria elegans* (Bonnem.) Schmitz and *Ptilota serrata* Kützting (Rogers *et al.* 1990), *Solieria filiformis* (Kützting) Gabrielson (Benevides *et al.* 1996) and *Enantiocladia duperreyi* (C. Agardh) Falkenberg (Benevides *et al.* 1998a). Algal lectins differ from higher plant lectins in a variety of properties. In general, algal lectins have lower molecular masses than most higher plant lectins and have no affinity for simple sugars but are more specific for complex oligosaccharides, often glycoproteins. Furthermore, most of marine algal lectins do not require divalent cations for their biological activity (Rogers & Hori 1993). They occur mainly in monomeric forms and have a high proportion of acidic amino acids, with isoelectric points from 4 to 6 (Shiomi *et al.* 1981, Hori *et al.* 1990). Compared to plant lectins, there are only a few reports on the use of marine algal lectins. For example, lectin extracts from some marine algae have shown to agglutinate strongly mouse FM3A tumor cells in lower concentrations than those required for lectins from land plants (Hori *et al.* 1986, 1987). Dalton *et al.* (1995) found that the pre-purified lectin of some marine algae exhibited high mitogenic activity for human lymphocytes. Furthermore, Griffin *et al.* (1995) demonstrated the use of *Codium fragile* (Suringar) Hariot lectin conjugated to colloidal gold as a new histochemical reagent. Following our continuous investigation of marine algal lectins, in the present work we describe the isolation and characterisation of a new lectin from the marine red alga *Pterocladia capillacea* (S.G. Gmel.) Santel. & Hommers.

Material and methods

The red alga *Pterocladia capillacea* (S.G. Gmel.) Santel. & Hommers. was collected from Pacheco beach (Fortaleza, Ceará, Brazil). After collection, the material was cleaned of epiphytes, washed with distilled water and stored at -20°C until use.

Human blood group A, B and O erythrocytes were collected from healthy donors at the Hematology Center, Federal University of Ceará (UFC). Rabbit, ox, goat and chicken erythrocytes were obtained by venous puncture of healthy animals.

The moisture content of the alga was determined by dehydration at 105°C for 24 h and the water content was estimated by difference. The ash was obtained by incineration of the dry alga at 600°C for 4 h, and the content was estimated by difference. Fat contents were determined as described by Triebold (1946). The Kjeldahl method (Baethgen & Alley

1989) was used for determination of alga total nitrogen. The protein content was calculated using a nitrogen conversion factor of 6.25. The carbohydrate content was estimated by difference.

In the purification procedure of the *P. capillacea* lectin, the alga was ground to a fine powder under liquid nitrogen, stirred continuously for 4 h with three volumes of 0.02 M Tris-HCl buffer, pH 7.5 (TB). The mixture was filtered through nylon, centrifuged at $10,000 \times g$ for 30 min at 4°C and finally, the liquid supernatant (crude extract) was precipitated by the addition of solid ammonium sulphate (0/80% saturation) for 4 h at 4°C . The precipitate was resuspended in buffer (F 0/80), dialysed against TB and then applied to a cross-linked guar-gum column equilibrated with the same buffer. After removing the unabsorbed material, the bound proteins were eluted from the column with 0.02 M glycine buffer, pH 2.6. The active fractions were pooled, dialysed, freeze-dried and submitted to gel filtration on a column of Sephadex G-100 that was equilibrated and eluted with TB.

The protein concentration was determined by the Bradford assay (Bradford 1976) using bovine serum albumin (BSA) as standard. The absorbance at 280 nm was used to estimate protein content in column eluates.

The neutral sugar content of the purified lectin was estimated by the phenol-sulphuric acid method (Dubois *et al.* 1956) using glucose as the standard.

The blood specificity from the *Pterocladia capillacea* lectin was determined by use of erythrocytes from different animals (rabbit, chicken, ox, goat) and humans from the ABO system, native and treated with the enzymes trypsin, bromelain, papain and subtilisin, according to Benevides *et al.* (1998a).

The hemagglutinating activity was assayed in the fractions obtained during the purification process according to Benevides *et al.* (1998a). In small glass tubes a series of 1:2 dilutions (0.1 mL) of the protein fractions was mixed with 0.1 mL of a 2% suspension of enzyme-treated erythrocytes. The degree of agglutination was monitored visually after the tubes had been left at 37°C for 30 min and subsequently at room temperature for another 30 min. The activity was expressed as hemagglutination units (H. U.). One H. U. was defined as the inverse of the highest dilution still capable of causing agglutination. Specific activity was expressed as hemagglutination units per mg of protein.

Inhibition tests were carried out using stock solutions (in 0.15 M NaCl) of sugars and glycoproteins. A two-fold dilution series was prepared for each substance in 0.15 M NaCl with a final volume of 0.1 mL. Aliquots (0.1 mL) of the diluted lectin were added to each tube of the diluted inhibitor series. The mixture was incubated at room temperature for 1 h, before the addition of the erythrocytes suspension (0.2 mL). The hemagglutination inhibition activity was recorded as the highest sugar dilution which inhibited the agglutinating activity.

To evaluate the effect of metal ions and EDTA on hemagglutinating activity, serial aliquots of two-fold dilutions of lectin solution were previously dialysed against 5 mM EDTA in 0.15 M NaCl for 16 h at 8 °C. The material was used for hemagglutination assays in the absence and presence of either 5 mM CaCl_2 or MnCl_2 . The hemagglutinating activity was measured by addition of trypsin-treated rabbit erythrocytes.

The buffers used to study the stability of *P. capillacea* lectin under different conditions of pH were: 0.02 M sodium acetate pH 5; 0.02 M sodium acetate pH 5 containing 0.15 M NaCl; 0.02 M sodium phosphate and sodium phosphate pH 7 containing 0.15 M NaCl; 0.02 M tris-HCl pH 7 and 0.02M tris-HCl pH 7 containing 0.15 M NaCl; 0.02 sodium borate pH 10 and 0.02 M sodium borate pH 10 containing 0.15 M NaCl.

The heat stability of the hemagglutinating activity of *Pterocladia capillacea* lectin was determined by incubation of aliquots of lectin solution at different temperatures (40, 50, 60, 70 or 80 °C) for 10, 20, 25 or 30 min and the remaining hemagglutinating activity determined. The activation energy of the denaturation process of the lectin was determined using the expression of Arrhenius (Dawes 1972).

The native molecular mass of the *Pterocladia capillacea* lectin was determined by gel filtration chromatography on a Sephadex G-100 column (1.6 × 89 cm) equilibrated and eluted with TB. Bovine serum albumin (66.0 kDa), carbonic anhydrase (29.0 kDa) and cytochrome C (12.4 kDa) were used as standard proteins. The void volume (V_o) was estimated with Blue Dextran (Sigma).

Descontinuous electrophoresis was carried out in a vertical system following the Laemmli method as described by Hames & Rickwood (1983). A 12.5% polyacrylamide slab in 0.025 M tris-HCl, 0.2 M glycine, pH 8.9, with 0.1% sodium dodecyl sulfate was used. Samples and standard were prepared in tris-HCl buffer, pH 6.8, containing SDS and β -mercaptoethanol. A standard picrate-Coomassie-blue method, as described by Stephano *et al.* (1986), was used for staining the gel following electrophoresis.

Results

The chemical composition, moisture, protein, oil, carbohydrate and ash of the red alga species studied are shown in table 1. Moisture contents value was 742.1 g.Kg⁻¹. The protein, oil, carbohydrate and ash contents were 254.0, 44.0, 691.9 and 10.1 g.Kg⁻¹ alga dry matter, respectively.

In the present study, it was found that the hemagglutinating activity in the protein fraction F 0/80 bound to a guar-gum column and eluted with 0.02 M glycine buffer, pH 2.6 (figure 1) showed a yield of 27,4% of the hemagglutinating activity corresponding to 14.5 times purification (table 2).

Table 1. Composition of the marine red alga *Pterocladia capillacea*. Means of triplicate determinations based on alga dry matter.

Constituent	g.Kg ⁻¹ alga dry matter
Moisture	742.1
Protein ¹	254.9
Carbohydrate ²	691.9
Lipid	44.0
Ash	10.1

1. Nitrogen × 6.25

2. Obtained by difference

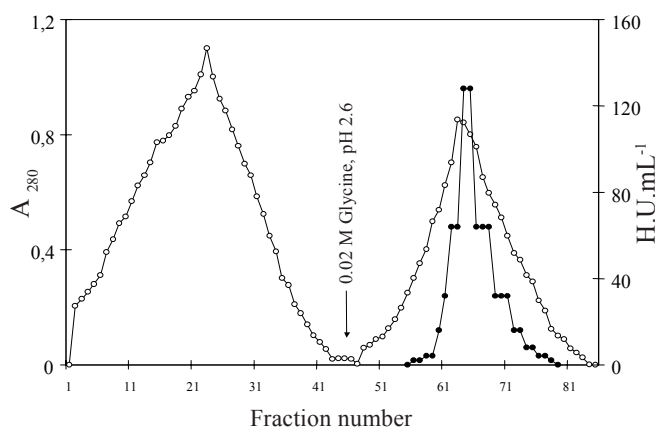


Figure 1. Affinity chromatography in guar gum column of the F 0/80 from the marine red alga *Pterocladia capillacea*. A column was previously equilibrated with buffer 0.02 M tris-HCl, pH 7.5. The first pick (PI) was eluted with the equilibrium buffer and the second pick (PII) eluted with buffer 0.02 M glycine-HCl pH 2.6 containing 0.15 M NaCl. A_{280} (○—○); H. U. (●—●).

The apparent molecular mass of the native lectin, determined by size-exclusion chromatography on a Sephadex G-100, was calculated to be 5.8 kDa. When the lectin was submitted to Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) in the presence of β -mercaptoethanol (figure 2) a single protein band was obtained, suggesting that the lectin could be a monomeric protein.

The lectin showed specificity for rabbit erythrocytes, when treated with the enzymes trypsin, bromelain, or subtilisin (table 3).

The results of sugar inhibition tests using a large number of simple sugars and glycoproteins for *P. capillacea* lectin are presented in table 4. The lectin did not show any inhibition by all simple sugars tested

Table 2. Purification of the lectin from the marine red alga *Pterocladia capillacea*.

Fraction	Total Protein (mg)	Total Activity* (H.U.)	Yield (%)	Specific Activity** (H.U.mgP ⁻¹)	MAC*** (µg.mL ⁻¹)	Purification (times)
Extract	172.0	220,160	100	1,280	0.80	1.0
F 0/80	67.2	164,480	74.7	2,447	0.40	1.9
Guar gum	3.3	61,440	27.4	18,618	0.05	14.5

* Inverse of the highest dilution capable of causing agglutination.

** Hemagglutination units per mg of protein.

*** Minimum agglutination capacity (minimum amount of protein that is able to agglutinate enzyme-treated rabbit erythrocytes)

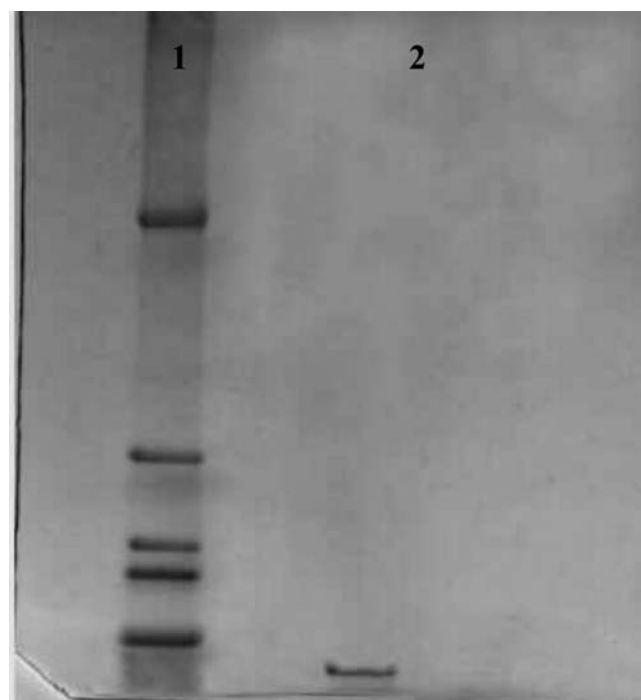


Figure 2. Electrophoresis in polyacrilamide gel in the presence and absence of SDS and β -mercaptoethanol. 1 - Standard proteins: lactoalbumin, 14.4 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30.0 kDa; Ovalbumin, 43.0 kDa and bovine serum albumin, 66.0 kDa. 2 - Purified lectin.

at 25 mM concentration. Of the glycoproteins tested, only avidin and porcine stomach mucin were inhibitory requiring the same concentration (156 µg.mL⁻¹). Fetuin and egg-white did not inhibit the lectin at maximum concentration tested (1,250 µg.mL⁻¹).

The hemagglutinating activity of the lectin was not affected by the presence of 5 mM EDTA, showing that the lectin is not a metallic protein.

The lectin was stable in the pH 7 to 10, retaining 50% of its haemagglutinating activity at pH 5. In

Table 3. Specificity of agglutination activity of blood cells of the lectin from the marine red alga *Pterocladia capillacea*. Red blood cells of chicken, ox, goat and human, A, B, O and AB groups were tested, but the haemagglutinating activity was not detected.

	Native	Trypsin	Bromelain	Papain	Subtilisin
Rabbit	-	64*	64	-	64

* Reciprocal of the highest dilution still giving a visible agglutination (H.U. . mL⁻¹).

- not detected.

addition, the hemagglutinating activity of *P. capillacea* lectin when submitted to heat treatment, was stable until 60 °C during 30 min, and still retained 50% of its original activity even after 30 min at 70 °C. When exposed at 80 °C during 10 min, the hemagglutinating capacity of the lectin declined rapidly, reaching 6% of the control value. The hemagglutinating activity was totally destroyed by heating the lectin at same

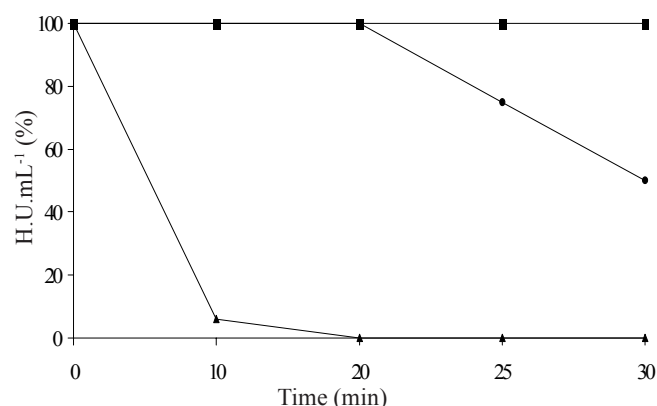


Figure 3. Effect of temperature on the activity of the lectin from the marine red alga *Pterocladia capillacea*. 40, 50 e 60 °C (■—■), 70 °C (●—●) e 80 °C (▲—▲).

Table 4. Inhibition of the haemagglutinating activity of the lectin from the marine red alga *Pterocladia capillacea*.

Glycoproteins	($\mu\text{g.mL}^{-1}$)*
Avidin	156
Porcine stomach mucin	156
Simple sugars**	0

* Minimum concentration ($\mu\text{g/mL}$) of glycoprotein able to inhibit the haemagglutinating activity

** D-glucosamine, D-mannose, D-ribose, L-arabinose, D-raffinose, D-melezitose, α -D-melibiose, L-rhamnose, D-arabinose, D-fructose, D-galactose, D-cellobiose, D-glucuronic acid, D-mannosamine, L-fucose, methyl α -D-mannopyranoside, sucrose, glucose, methyl α -D-glucopyranoside, D-xylose, trehalose, salicin were non-inhibitory at 25 mM concentration.
Fetuin and egg-white were non-inhibitory at 1250 $\mu\text{g.mL}^{-1}$ concentration

temperature for 30 min. (figure 3). The activation energy of denaturation process (ΔG^\ddagger) was estimated to be 106.87 kJ.mol⁻¹.

Carbohydrate analysis by the phenol-sulphuric acid method showed that the lectin does not possess carbohydrate in the structure.

Discussion

The high content of water is a peculiar characteristic in the diverse classes of algae studied, probably due to the necessity of a thermal regulator mechanism of these organisms. Among the organic constituents the carbohydrates was the highest one. The results show that this alga species can be regarded as a protein source, comparable to values found to various leguminous seeds (Singh & Singh 1992) and, consequently a lectin source.

Cross-linked guar-gum, a galactomannan consisting of chains of (1→4) linked β -D-manose with α -D-galactose linked (1→6) as single unit side chains, has been used as an efficient, inexpensive and rapid general affinity medium for the purification of lectins from land plants (Sampaio *et al.* 1998b). The utilisation of affinity chromatography is also an important tool in the process of purification of algae lectins. Many lectins from these vegetables were isolated by this technique, such *Ptilota filicina* J. Agardh (Sampaio *et al.* 1998b), *Enantiocladia duperreyi* (Benevides *et al.* 1998a) and *Caulerpa cupressoides* (Benevides *et al.* 2001). Most of the lectins isolated from marine red algae have low molecular weight (Rogers & Hori 1993). The marine red alga *Hypnea japonica* Tanaka contains four lectins

with molecular weights of 4.2-12.0 kDa (Hori *et al.* 1986). The lectin from the red alga *Bryothamnion seaforthii* (Turner) Kützinger exhibited a molecular mass of 3.5 kDa (Ainouz *et al.* 1995), while the lectin from *Bryothamnion triquetrum* (Gmelin) Howe displayed a molecular mass of 4.5 kDa (Calvete *et al.* 2000).

The hemagglutination inhibition studies carried out with purified *Pterocladia capillacea* lectin, revealed that the lectin is not inhibited by simple sugars but by glycoproteins. This is in general agreement with those found for the numerous marine algal lectins, such as *Cystoclonium purpureum* (Huds.) Batters (Kamiya *et al.* 1980), *Solieria chordalis* (C. Agardh) J. Agardh (Rogers & Topliss 1983), *Plumaria elegans* and *Ptilota serrata* (Rogers *et al.* 1990), *Gracilaria bursa-pastoris* (Gmelin) Silva (Okamoto *et al.* 1990), *Solieria filiformis* (Benevides *et al.* 1996) and *Gracilaria verrucosa* (Hudson) Papenfus (Kakita *et al.* 1997). The inhibition observed with porcine stomach mucin and avidin may be due to their complex sugar moieties and not due to their terminal residue alone, since that porcine stomach mucin is a glycoprotein with a terminal GalNAc residue, and fucose and galactose as internal residues (Slomiany & Mayer 1972). Algal lectins are, in general, more specific for complex oligosaccharides often glycoproteins (Rogers & Hori 1993). Therefore, the inhibition of the hemagglutinating activity from the marine algae lectins by glycoproteins was also observed in some marine algal, such as *Agardhiella tenera* Schmitz (Shiomi *et al.* 1979), *Ulva lactuca* (Sampaio *et al.* 1998a), *Bryothamnion seaforthii* (Turner) Kützinger and *B. triquetrum* (Ainouz *et al.* 1995), and *Amansia multifida* Lamouroux (Costa *et al.* 1999).

Like most lectins from marine red algae (Rogers & Hori, 1993), the *Pterocladia capillacea* lectin does not require divalent cations for the maintenance of its biological activity, since the addition of EDTA to the reaction medium did not affect the haemagglutinating activity, suggesting that this lectin is not a metallic protein. However, the lectins from the red algae *Ptilota serrata* (Rogers *et al.* 1990), *Ptilota filicina* (Sampaio, 1998b), *Enantiocladia duperreyi* (Benevides *et al.* 1998a) and from the green algae *Ulva laetevirens* Areschoug (Sampaio *et al.* 1996) and *Ulva lactuca* (Sampaio *et al.* 1998a) exhibited dependence of metals such as Ca²⁺, Mn²⁺ and Mg²⁺, as is the case with most plant lectins. In accordance with Rogers & Hori (1993) lectins from the red algae do not require divalent cations for their biological activity.

The hemagglutinating activity from the *Pterocladia capillacea* lectin was affected only by

exposure to a temperature of 70 °C for 25 min. The activation energy of denaturation (ΔG°) was estimated to be 106.87 kJ.mol⁻¹ which is similar to the value found for some plant lectins (Cavada *et al.* 1996) and marine algae (Benevides *et al.* 1998a). The absence of carbohydrate in the structure of the lectin differ of the observed to another lectins from marine algae: *Codium tomentosum* (Huds) Stackhouse (Fabregas *et al.* 1988), *Bryothamnion seaforthii* and *Bryothamnion triquetrum* (Ainouz *et al.* 1995), *Solieria filiformis* (Kützting) Gabrielson (Benevides *et al.* 1996), *Enantiocladia duperreyi* (Benevides *et al.* 1998a) and *Caulerpa cupressoides* (Benevides *et al.* 2001).

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