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ORIGINAL PAPER

Haemagglutinin of the antarctic seaweed *Georgiella confluens* (Reinsch) Kylin: isolation and partial characterization

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Abstract The marine red alga *Georgiella confluens* collected from Mackellar Inlet, King George Island, South Shetland Islands, Antarctic, was used in the isolation of a protein with agglutinating activity. The *Georgiella confluens* haemagglutinin (GCH) was extracted with 20 mM phosphate buffer, pH 7.0, and purified through ion exchange chromatography, followed by affinity chromatography on immobilized porcine stomach mucin. Among the erythrocytes analysed (human A, B and O groups, rabbit and chicken), GCH agglutinated specifically chicken erythrocytes. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the haemagglutinin revealed a single band of 21.5 kDa, while by gel filtration on Sephadex G-100 its native molecular mass was 25.5 kDa, suggesting that GCH is a monomeric protein. Haemagglutination studies showed that the GCH activity was stable through temperature variations and did not exhibit divalent cation dependence. Furthermore, the haemagglutinin was inhibited by the complex glycoproteins of porcine stomach mucin and fetuin, whereas the mono-, di-, and trisaccharides tested showed no effect.

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Introduction

Haemagglutinins are biomolecules widely distributed in nature and constitute a group of proteins or glycoproteins present in a range of organisms from bacteria to animals. They are carbohydrate-binding proteins associated with important effects such as cell aggregation and glycoconjugate precipitation (Liener et al. 1986; Peumans and Van Damme 1998), and therefore can be used as carbohydrate probes, making them useful tools in a variety of biochemical and biomedical research areas (Van Damme et al. 1998). Since the first report of the occurrence of haemagglutinin in seaweeds (Boyd et al. 1966), several studies describing the purification of these proteins have been developed (Rogers et al. 1980; Fabregas et al. 1985; Hori et al. 1988, 1990; 2000 Ainouz and Sampaio 1991; Sampaio et al. 1998a, b, c, 2002; Kawakubo et al. 1999; Nagano et al. 2002; Ambrosio et al. 2003); however, the amount of purified and characterized proteins is still small in comparison to those from land plants and also they differ from each other in a variety of properties. In general, algal haemagglutinins are monomeric and low molecular weight proteins that do not require divalent metal cations for their biological activities and are more specific for complex oligosaccharides, often glycoproteins, than for simple sugars (Shiomi et al. 1981; Hori et al. 1990; Rogers and Hori 1993). To date, only six complete amino acid sequences from algal haemagglutinins have been determined (Calvete et al. 2000; Hori et al. 2000; Wang et al. 2004; Nagano et al. 2005), and their sequence shows no similarity to any known plant haemagglutinin.

In 2007, another study by our group (Souza et al. 2007) reported the first screening for haemagglutinin activity in aqueous extracts from 18 algae collected on King George Island, South Shetland Islands, Antarctic. Analyses of the active fractions included haemagglutination inhibition studies with a selection of sugars and glycoproteins.

In the present work, the isolation and partial characterization of the first haemagglutinin (GCH—*Georgiella confluens* haemagglutinin) of a marine alga from the Antarctic continent is described.

Materials and methods

Algal material

The red alga *Georgiella confluens* was collected from the Mackellar Inlet (62° 05′ 252″ S and 58° 25′ 137″ W), King George Island, South Shetland Islands, Antarctic. After collection, the material was transported to Brazil, cleaned in order to remove epiphytes, washed with distilled water and stored at −20°C until used. The algal material was identified by Professor Andres Mansilla from Departamento de Ciencias y Recursos Naturales, Universidad de Magallanes, Chile and exsiccates of *G. confluens* were deposited in the herbarium of the Universidad de Magallanes, Chile.

Blood cells

Human blood samples (ABO system) were obtained from healthy donors at the Haematology Center of the Universidade Federal do Ceará (UFC). Rabbit red blood cells were obtained by venous puncture from healthy animals reared at Departamento de Bioquímica e Biologia Molecular, UFC. Chicken blood was obtained from healthy animals reared at the Departamento de Zootecnia, UFC.

Haemagglutinin purification

Algal material was ground to a fine powder in a mortar and pestle in the presence of liquid nitrogen. For extraction, the powder was stirred with 3 volumes of 20 mM phosphate buffer, pH 7.0 (buffer A) for 18 h at 4°C. Insoluble algal material was removed by filtration using a nylon membrane, followed by centrifugation at 14.000 g for 30 min at 4°C. For purification of GCH, the crude extract was partially concentrated by lyophilization, dialysed against buffer A and loaded onto a DEAE-Sephacel column (1.6 × 35 cm) equilibrated with the same buffer. The adsorbed proteins were eluted with buffer A containing 1.0 M NaCl at a flow rate of 60 mL/h. The elution was monitored at 280 nm, while 3 mL fractions were collected manually and tested for haemagglutinating activity. Active fractions (PII-DEAE) were pooled, dialysed extensively against phosphate-buffered saline (PBS), and further purified by affinity chromatography on a column (1.8 × 5.0 cm) of mucin-Sepharose 4B. The column was equilibrated with PBS, and unbound proteins were removed by elution with the equilibrium buffer. The retained haemagglutinin was then desorbed from the matrix by the addition of buffer B (0.1 M glycine and 0.15 M NaCl, pH 9.6) in a pure form and named as GCH. Fractions showing haemagglutinating activity were pooled, dialysed against distilled water, freeze-dried and stored at 4°C until use.

Protein and carbohydrate quantification

Absorbance was measured at 280 nm (spectrophotometer Femto 700, Brazil) to determine the protein amount obtained during the purification process and to monitor elution profiles during chromatography. Protein quantification was developed using the method described by Bradford (1976) with bovine serum albumin (BSA) used as standard.

The amount of neutral sugars in the purified haemagglutinin was estimated by the phenol–sulfuric acid method (Dubois et al. 1951), using glucose as standard.

Erythrocyte specificity and hapten-inhibition tests

The erythrocyte specificity and hapten-inhibition assays with carbohydrate and glycoproteins were performed as described previously by Benevides et al. (1998). Carbohydrates, proteolytic enzymes and BSA were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, Missouri, USA). The blood specificity of the GCH was determined using erythrocytes from rabbit, chicken and humans from the ABO system, native or treated with the enzymes trypsin, bromelain, papain and subtilisin. Haemagglutinating activity was assayed with the crude extract and the fractions obtained during the purification process. Samples to be tested (0.1 mL) were assayed in small glass test tubes using twofold serial dilutions in PBS containing 0.15 M NaCl. A 2% erythrocytes suspension was added to each tube (0.1 mL), and the agglutination degree was monitored visually after the tubes had been left at 37°C for 30 min followed by another 30-min interval at room temperature. The haemagglutination titre (HU mL^{-1}) was recorded as the reciprocal of the highest dilution still giving visible agglutination. Specific activity was expressed as haemagglutination units (HU) per milligram of soluble protein (HU mg P^{-1}).

Hapten-inhibition tests were carried out using stock solutions of sugars (*N*-acetyl-D-Glucosamine, *N*-acetyl-D-Galactosamine, L(+) arabinose, D(−) cellobiose, D(+) glucosamine, D(+) glucose, D(+)mannose, methyl- α-D-galactopyranoside, D(+) raffinose, D(+) rhamnose, Salicine,

D(+) xilose, D(+) fructose) and glycoproteins (fetuin, porcine stomach mucin, bovine submaxillary mucin and Mannan from *Saccharomyces cerevisiae*). Twofold serial dilutions of sugar (100 mM) or glycoprotein (500 μg mL^{-1}) solutions were prepared in 0.15 M NaCl with a final volume of 0.1 mL. An equal volume of the crude extract (1 mg of protein/mL) was added to each tube and the mixture allowed to interact for 1 h at room temperature, before addition of the of trypsin-treated chicken erythrocytes (0.2 mL). This mixture was incubated at 37°C for 30 min followed by another 30 min interval at room temperature. The lowest concentration of a specific sugar or glycoprotein that inhibited haemagglutination (minimum inhibitory concentration, MIC) was recorded and used to define inhibitory potency.

Effects of EDTA and divalent cations (Ca^{2+} and Mn^{2+}) on GCH haemagglutinating activity

The purified active haemagglutinin was dialysed against 5 mM EDTA (ethylenediaminetetraacetic acid) in 0.15 M NaCl for 16 h at 4° C and then submitted to haemagglutination activity tests as previously described. The haemagglutination assays were carried out both in the presence and absence of the divalent cations Ca^{2+} and Mn^{2+}. This was achieved by submitting the EDTA-treated haemagglutinin to twofold serial dilutions in 0.15 M NaCl with or without 5 mM $CaCl_2$ and 5 mM $MnCl_2$. The haemagglutinating activity was measured by the addition of trypsin-treated chicken erythrocytes (Lima et al. 2005).

Heat stability of haemagglutinin

The heat stability of GCH was determined by incubating aliquots of a haemagglutinin solution (2 mg/mL) at 40, 50, 60, 70, 80 and 90°C for 10, 20, 25 and 30 min. The samples were then cooled and assayed for haemagglutinating activity as described previously. The free energy change ($\Delta G'$) of activation of the haemagglutinin denaturation process was determined using the Arrhenius expression. The velocity constant of the reaction (k_1) was first determined as the slope of the curve obtained by the expression $k_1 t = -\ln A/A_0$, where A is the residual haemagglutinating activity after heat treatment, A_0 is the initial haemagglutinating activity before heat treatment and t is the time of heat treatment (in seconds). The velocity constant $k1$ is related to the standard free energy change by the following formula: ($\Delta G' = RT \ln(kT/k_1 h)$, where R is the gas constant (1.987 cal mol^{-1} $°K^{-1}$), T is the absolute temperature (°K), k is the Boltzmann constant (1.37×10^{-16} erg $°K^{-1}$), k_1 is the velocity constant and h is Planck's constant (6.25×10^{-27} erg s^{-1}) (Leite et al. 2005).

Gel electrophoresis and molecular mass determination

The native molecular mass of GCH was determined by gel filtration chromatography on a Sephadex G-100 column (1.6 × 63 cm) equilibrated and eluted with PBS. Bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) were used as standard proteins. The void volume (Vo) was estimated with Blue Dextran (Sigma Chemical Co.). A calibration curve was constructed by plotting elution volume (Ve) over void volume (Vo) against the logarithm of the molecular weight (MW) of the standard proteins.

Molecular mass estimation under denaturing conditions was carried out through discontinuous electrophoresis using a vertical system following the Laemmli method (SDS–PAGE) as described by Hames and Rickwood (1981). A 12.5% polyacrylamide in 0.025 M Tris–HCL, 0.2 M glycine, pH 8.9, with 0.1% (w/v) sodium dodecyl sulfate was used. Samples and standards were prepared in Tris–HCl buffer, pH 6.8, containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol, followed by heating at 100°C for 10 min. BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.2 kDa) were used as standard proteins. A standard Coomassie blue method was used for staining protein bands following electrophoresis (Stephano et al. 1986).

Results

Crude extract of the red alga *G. confluens* was able to agglutinate non-enzyme-treated and trypsin-treated chicken erythrocytes (16 and 128 HU mL^{-1}, respectively), while no agglutination was observed against rabbit or human erythrocytes, even when cells were treated with the proteolytic enzymes (data not shown). The carbohydrate-binding specificity of the GCH was studied through the incubation of crude extract with various sugars and glycoproteins before development of the haemagglutination assay (Table 1). Among the glycoproteins tested, only fetuin and porcine stomach mucin were inhibitory at the concentration of 2.5 μg mL^{-1}, while bovine submaxillary mucin and mannan from *Saccharomyces cerevisiae*, as well as all the simple sugars tested presented no effect on GCH activity at the maximum concentration used (500 μg mL^{-1} and 100 mM for glycoproteins and simple sugars, respectively).

The crude extract of *G. confluens* was submitted to ion exchange chromatography on a DEAE-Sephacel column. The extract was initially applied to the matrix and the non-retained fraction (PI-DEAE), devoid of haemagglutinating activity, was first eluted with the equilibrium buffer,

Table 1 Hapten-inhibition tests of *Georgiella confluens* haemagglutinin

Glycoproteins and simple sugars	Minimum inhibitory concentration (MIC)[a]
Fetuin	2.5 μg mL^{-1}
Bovine submaxillary mucin	NI
Porcine stomach mucin	2.5 μg mL^{-1}
Mannan from *Saccharomyces cerevisiae*	NI
Simple sugars[b]	NI

NI no inhibition

[a] Lowest concentration of a specific glycoprotein or simple sugar that inhibited one haemagglutinating unit (HU) of purified GCH when assayed with trypsin-treated chicken erythrocytes

[b] *N*-acetyl-D-Glucosamine, *N*-acetyl-D-Galactosamine, L(+) arabinose, D(−) cellobiose, D(+) glucosamine, D(+) glucose, D(+)mannose, methyl-α- D-galactopyranoside, D(+) raffinose, D(+) rhamnose, Salicine, D(+) xilose, D(+) fructose

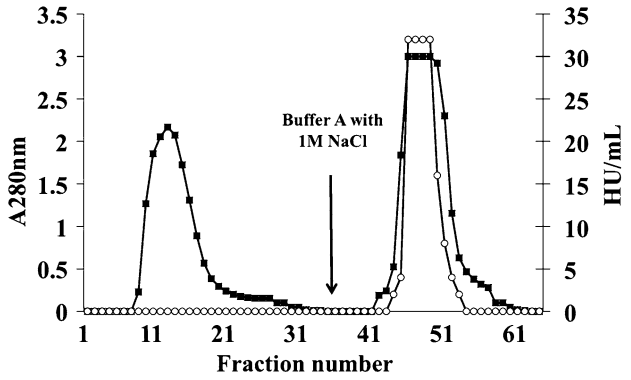

Fig. 1 Ion exchange chromatography of the crude extract of *Georgiella confluens*. The column was equilibrated with buffer A (20 mM phosphate buffer, pH 7.0). The unbound proteins were washed from the column with the same buffer. The adsorbed proteins were eluted with buffer A containing 1 M NaCl; (*filled square*) absorbance at 280 nm, (*circle*) haemagglutinating activity

followed by a second peak (PII-DEAE) eluted with buffer A containing 1.0 M NaCl, that showed haemagglutinating activity against the chicken trypsin-treated erythrocytes (Fig. 1). The yield of protein from the active fraction (PII-DEAE) was 22.5% and with respect to the specific activity observed in total extract, this represents a purification of 3.0 times (Table 2).

Once the activity of the haemagglutinin present in the crude extract was inhibited by porcine stomach mucin, the active fraction from ion exchange chromatography (PII-DEAE) was dialysed and then submitted to affinity chromatography on a mucin-Sepharose column. As shown in Fig. 2, the applied sample was eluted in two peaks where the first one, PI-mucin, did not show haemagglutinating activity, and the second peak (PII-mucin), adsorbed to the matrix and eluted with 0.1 M glycine buffer pH 9.6 containing 0.15 M NaCl, including all the haemagglutinating activity initially present in the active fraction loaded on the column. As shown in Table 2, the haemagglutinin from *G. confluens* (PII-mucin) showed a protein yield of 5% and a purification of 9.7 times.

Divalent cations do not seem to be required for GCH activity as the haemagglutination titre of EDTA-treated haemagglutinin did not show any significant difference in relation to the native protein. Moreover, the haemagglutinating activity of GCH submitted to heat treatment remained stable until 40 and 50°C during 30 min, and retained 50% of its original activity even after 30 min at 60, 70 and 80°C. When exposed to 90°C for 10 min, the agglutinating capacity of the haemagglutinin declined rapidly, reaching 13% of the initial value. The haemagglutinating activity was totally destroyed by heating the GCH at the same temperature for 20 min (Fig. 3). The activation energy of the denaturation process ($\Delta G'$) was estimated to be 19.7 kcal/mol. Carbohydrate analysis by the phenol–sulphuric acid method showed that the haemagglutinin possesses carbohydrate in its structure. SDS–PAGE of the purified haemagglutinin in the presence of 2-mercaptoethanol revealed only one band of protein corresponding to a M*r* of 21.5 kDa (Fig. 4). The apparent molecular mass of the native GCH determined by gel filtration on Sephadex G-100 was approximately 25.5 kDa (Fig. 5).

Table 2 Purification of the haemagglutinin from the marine red alga *Georgiella confluens*

Fraction	Volume (mL)	Total protein (mg)	Yield (%)	Total activity[a] (Total HU)	Specific activity[b] (HU mg P^{-1})	Purification (times)	MAC[c] (μg)
Crude extract	20	36	100	2,560	71.1	1.0	14.06
PII—DEAE	54	8.1	22.5	1,728	213.3	3.0	4.68
PII—Mucin	12	1.8	5.0	384	691.2	9.7	4.68

[a] Total haemagglutination units (total activity) is the volume × haemagglutination titer (HU mL^{-1})

[b] Haemagglutination units per mg of protein

[c] Minimum agglutination capacity (minimum amount of protein that is able to agglutinate trypsin-treated chicken erythrocytes)

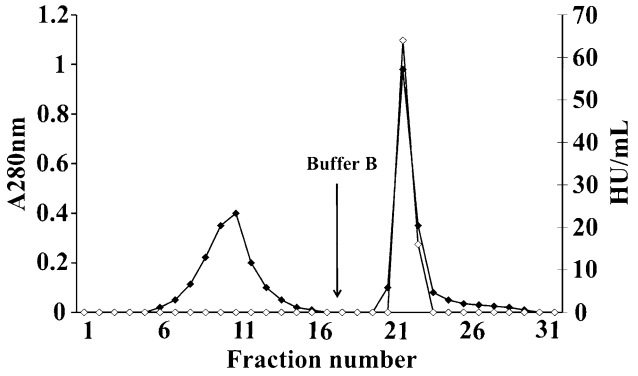

Fig. 2 Affinity chromatography of the retained peak from the ion exchange chromatography (PII-DEAE) of *Georgiella confluens* on a mucin-Sepharose 4B column. The column was equilibrated with phosphate-buffered saline, pH 7.0, and the unbound proteins were washed with the same buffer. The adsorbed haemagglutinin was eluted with buffer B (0.1 M glycine and 0.15 M NaCl, pH 9.6); (*filled diamond*) absorbance at 280 nm, (*diamond*) haemagglutinating activity

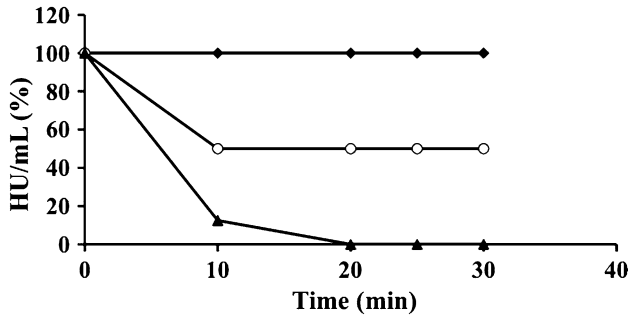

Fig. 3 Effect of temperature on the haemagglutinating activity of *Georgiella confluens* haemagglutinin; (*filled diamond*) 40 and 50°C, (*circle*) 60, 70 and 80°C, (*filled triangle*) 90°C. Residual haemagglutinin activity across heat treatments is shown as the percentage of haemagglutinating units (HU) at each time in relation to the haemagglutinating titer (HU mL^{-1}) of the native protein

Discussion

The erythrocyte specificity of the GCH was evaluated through haemagglutination assays against rabbit, chicken and human red cells. GCH was able to agglutinate only chicken erythrocytes; however, rabbit erythrocytes, which are usually described as susceptible for agglutination by algae extracts (Chiles and Bird 1989; Ainouz and Sampaio 1991; Ainouz et al. 1992; Rogers and Hori 1993), did not react with the haemagglutinin. These results are in agreement with work of other authors (Shiomi et al. 1981; Leite et al. 2005; Lima et al. 2005) who have studied haemagglutinins from the genus *Gracilaria*. To improve the interaction of the haemagglutinin with carbohydrates present on the surface of red cells, proteolytic enzymes were used. According to Lis and Sharon (1986), this treatment promotes the removal of sialoglycoproteins and their negative charge associated with the cell surface and reduces steric hindrance by removing polypeptides, thus producing

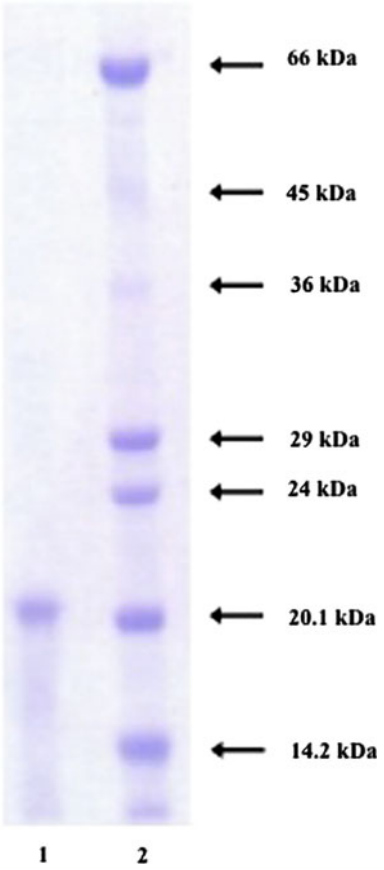

Fig. 4 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified haemagglutinin from *Georgiella confluens*. Lane 1—haemagglutinin treated with 2-mercaptoethanol; lane 2—standard proteins (α—Lactoalbumin 14.2 kDa; Soybean trypsin inhibitor, 20.1 kDa; Trypsinogen, 24 kDa; Carbonic anhydrase, 29 kDa; Glyceraldehydes-3-phosphate dehydrogenase 36 kDa; Ovalbumin, 45 kDa, Bovine serum albumin, 66 kDa)

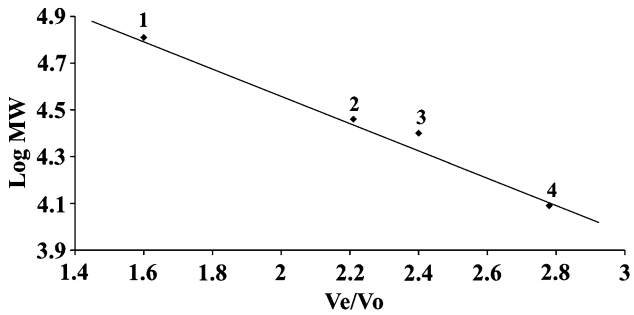

Fig. 5 Molecular mass estimation of *Georgiella confluens* haemagglutinin by gel filtration chromatography. Purified haemagglutinin and standards were loaded on Sephadex G-100. Standard proteins were the following: (*1*) Bovine serum albumin (66 kDa); (*2*) Carbonic anhydrase (29 kDa) and (*4*) Cytochrome c (12.4 kDa); (*3*) GCH. Ve (elution volume); Vo (void volume); Logarithm of the molecular weight (log MW)

greater exposure of new groups of carbohydrates and glycoconjugates present in the cell membrane, facilitating recognition by the haemagglutinin and improving its agglutination efficiency. This probably explains the increase in the haemagglutination titre when the chicken erythrocytes were treated with trypsin.

GCH haemagglutinating activity was inhibited by the glycoproteins fetuin and porcine stomach mucin. Fetuin is a complex *N*-linked glycoprotein containing units of the disaccharide Galβ4GlcnAc (*N*-acetyllactosamine) substituted with sialic acid, attached to the pentasaccharide core (Baenziger and Fiete 1979; Gabius and Gabius 1997), while porcine stomach mucin is an *O*-linked glycoprotein with terminal GalNAc residues, as well as fucose and galactose as internal residues (Slomiany and Meyer 1972; Leite et al. 2005). On the other hand, GCH was not inhibited by bovine submaxillary mucin, a glycoprotein having *N*-acetyl neuraminic acid (NeuNAc) as a terminal residue linked to GalNAc. Thus, the carbohydrate-binding specificity of GCH is complex and possibly this haemagglutinin reacts with structures that are more complex than monosaccharides. In fact, Rini (1995) reported that larger, more complex polysaccharides interact with secondary sites on the haemagglutinins surface as well as with the primary binding site; however, fine sugar specificity studies are necessary for a better understanding of the characteristics of GCH.

The GCH was successfully purified through ion exchange chromatography, followed by affinity chromatography on immobilized porcine stomach mucin. Among haemagglutinins isolated from seaweeds, the technique of ion exchange chromatography has proven to be extremely useful as an initial stage of purification, separating protein peaks with different charges and thereby contributing to a more satisfactory result (Costa et al. 1999), while affinity chromatography, in which the ligand is recognized and bound by the haemagglutinin, has become over the years a simple and effective process in the isolation of this class of proteins. In fact, this type of chromatography has also been used to isolate some of red seaweed haemagglutinins, namely the ones from *Solieria chordalis* (Rogers and Topliss 1983), *Carpolpetis flabelata* (Hori et al. 1987), *Solieria filiformis* (Benevides et al. 1996), *Gracilaria bursa-pastoris* (Okamoto et al. 1990), *Enantiocladia duperreyi* (Benevides et al. 1998), *Ptilota filicina* (Sampaio et al. 1998a), *Gracilaria ornata* (Leite et al. 2005) and *Vidalia obtusiloba* (Melo et al. 2004).

Results from molecular mass determination studies suggest that *G. confluens* haemagglutinin is a monomeric protein, since close values, 25.5 kDa and 21.5 kDa, were obtained for the molecular mass of GCH when evaluated in its native form through gel filtration chromatography and by SDS–PAGE in the presence of 2-mercaptoethanol, respectively. These results also show that GCH may have a low molecular weight, similar to other algal haemagglutinins already described. For example, haemagglutinins from *Gracilaria ornata, Amansia multifida* and *Enantiocladia duperreyi* with 17.36, 26.9 and 24.7 kDa, respectively (Benevides et al. 1998; Costa et al. 1999; Leite et al. 2005).

Published data show that agglutinating activities of some seaweed haemagglutinins are lost after dialysis in the presence of the chelating agent EDTA (Benevides et al. 1998; Sampaio et al. 1998a, b; Melo et al. 2004). However, this same treatment did not alter the agglutinating activities of GCH and similar behaviour has been observed for *Gracilaria ornata* (Leite et al. 2005), *Gracilaria cornea* (Lima et al. 2005) and *Eucheuma serra* (Kawakubo et al. 1997) haemagglutinins.

Several red algae haemagglutinins studied have been considered thermosensitive (Shiomi et al. 1979; Kamiya et al. 1980; Hori et al. 1987; Melo et al. 2004), with total or almost total activity loss when heated to temperatures equal to or above 60°C, the thermostability results demonstrated that GCH is thermo-resistant, similar to other red algae haemagglutinins, such as *Pterocladiella capillacea* (Oliveira et al. 2002), *Enantiocladia duperreyi* (Benevides et al. 1998), *Amansia multifida* (Costa et al. 1999) and *Gracilaria ornata* (Leite et al. 2005). The results obtained for GCH are in accordance with the information usually found in related works described in literature for other haemagglutinins from Rhodophytas. According to Rogers and Hori (1993), red algae haemagglutinins are generally low molecular weight molecules that recognize glycoproteins but not monosaccharides and do not require divalent cations to exercise their activity.

In conclusion, this work reports the isolation of the first haemagglutinin from an alga from the Antarctic continent. GCH, purified by affinity chromatography on a mucin-Sepharose matrix, is a monomeric protein with a molecular mass of approximately 25.5 kDa. Haemagglutinating activity was only inhibited by glycoproteins, was stable across temperature variations and did not exhibit divalent cation dependence.

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