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A new survey of Brazilian marine algae for agglutinins

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Abstract

Aqueous protein extracts from 30 Brazilian marine algae were examined for haemagglutinating activity using native and enzyme-treated rabbit, chicken, sheep and human erythrocytes. Most extracts agglutinated at least one of the blood cells used. Sheep and rabbit erythrocytes were more suitable for detection of the agglutinating activity. The minimum protein concentration necessary to produce positive agglutination was usually lower with enzyme-treated erythrocytes than native ones. The five algal protein extracts showing the greatest haemagglutination titre were tested for sugar-binding specificity. Only the activity present in the green alga *Caulerpa cupressoides* was inhibited by simple sugars and not by the glycoproteins tested. The activity of the other four extracts was inhibited by at least one of the glycoproteins utilised.

Introduction

The increasing interest in marine natural products chemistry has led to the discovery of new biologically active compounds and marine algae have been subjected to increasing study for this purpose (Amico, 1995). They have been reported to contain high amounts of water-soluble macromolecules such as polysaccharides, proteins, glycoproteins and other less polar compounds of low molecular weight, some of them exhibiting particular biological properties *in vitro*.

Biochemical experiments based on agglutinating tests have revealed the presence of haemagglutinating activity in many algal extracts against erythrocytes from several animal species. In most studies this haemagglutinating activity is referred to the presence of proteins or glycoproteins having specificities for carbohydrate structures binding selectively to red blood cells and microorganisms. These proteins, which are found in a variety of organisms, were reported to exhibit in the sap of some marine algae (Boyd et al., 1966). Following this pioneering work, other workers have demonstrated that lectins are present in many algal species e.g Blunden et al., 1975; Rogers et al., 1980; Muñoz et al., 1987; Hori et al., 1988. Although considerable progress has been made in understanding the biochemical character of lectins, little is known about their biological role in nature. Hori et al. (1988) suggested that lectins may play a common, but as yet unknown, physiological function in marine algae.

The carbohydrate specificity of lectins has made them attractive proteins. This property has enabled them to become useful tools for various scientific purposes including detection and identification of blood groups and microorganisms, mitogenic stimulation of immune cells, determination of carbohydrates in solutions, on macromolecules and cells, purification of glycoproteins and cell fractionation and as a tool for taxonomy. They have also been used as molecular probes for histochemical studies; in the case of algae Griffin et al. (1995) first demonstrated that *Codium fragile* lectin conjugated to gold particles may be used as a histochemical reagent.

Our studies on Brazilian marine algae for haemagglutinins have screened 47 species using native and enzyme-treated human and animal erythrocytes (Ainouz & Sampaio, 1991; Ainouz et al., 1992). Some of these lectins have been isolated and characterized in detail and show unique properties such as low molecular weight (Ainouz et al., 1995; Benevides et al., 1996).

The present study reports screening for lectin activity in aqueous extracts from a further 30 algae collected on the Northeast coast of Brazil. Analyses are included using competitive inhibition experiments of the binding affinity for simple sugars and glycoproteins of the active fractions.

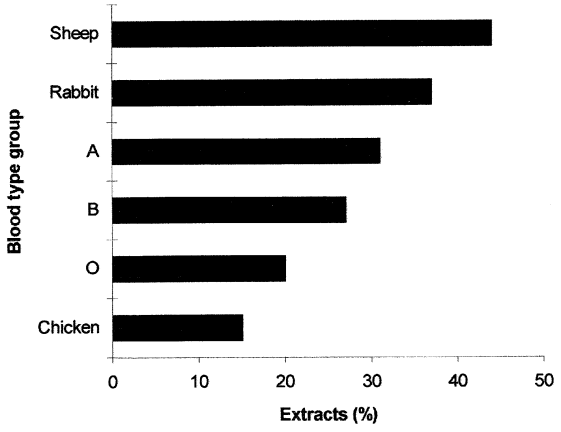

Figure 1. Percentage distribution of hemagglutinating activity among the marine algae studied.

Materials and methods

Chemicals

Carbohydrates, proteolytic enzymes and bovine serum albumin (BSA) were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, Missouri, USA) at the highest purity available. All other reagents used were of analytical grade.

Marine algae

All species were collected on the Northeast coast of Brazil (Pacheco and Flexeiras beaches, State of Ceará) during January to December 1995. Upon collection, the algae were cleaned of epiphytes, rinsed with tap water, and stored at -20 °C before utilisation.

Extraction and haemagglutination assays

Crude extracts were prepared by homogenisation with 3 volumes of 0.85% NaCl. After centrifugation at $10,000 \times g$ for 30 min at 4 °C the supernatants were stored at -20 °C until used. Haemagglutination tests were carried out with erythrocytes from human and various animals in a native state or enzyme-treated with trypsin, bromelain, papain and subtilisin, following standards procedure (Ainouz & Sampaio, 1991; Ainouz et al., 1992). Rabbit, chicken and sheep blood cells were obtained from the animal by venous puncture and human blood groups A, B and O were obtained from the Center of Haematology of Ceará, Brazil. For each blood type tested, serial two-fold dilutions of the algal extracts were prepared using 0.85% NaCl. An equal volume of the 2% erythrocyte suspension was added to each dilution. The tubes were gently shaken and left for 1 h at room temperature, after which time the degree of macroscopic agglutination was observed. The greatest dilution that could agglutinate the erythrocytes, the haemagglutination titre, was defined as containing one haemagglutinating unit per ml. The activity of the algal extract was recorded as the minimum amount of protein that caused agglutination. Protein concentrations were determined by the method of Lowry et al. (1951) using BSA as standard.

Haemagglutination inhibition assay

Inhibition studies were performed using five algal protein extracts: *Gracilaria foliifera, G. curtissiea, Agardhiellla ramosissima, Caulerpa cupressoides* and *Heterodasya sertularioides*. The inhibition assay was carried out following standard procedures as described by Ainouz et al. (1995), using the following substances: simple sugars: D(+) glucose, D(+) galactose, D(−) fructose, L(+) arabinose, D(−) arabinose, D(+) raffinose, L(+) rhamnose, D(+) mannose, D(−) cellobiose, D(+) xilose, D(+) glucosamine, N-acetylglucosamine, N-acetylgalactosamine, salicine, lactose; glycoproteins: avidin, egg white, fetuin, porcine stomach mucin and yeast mannan. The results were expressed as the minimum concentration of simple sugar or glycoproteins that caused inhibition of the haemagglutinating activity using rabbit enzyme-treated erythrocytes as indicator cells.

Table 1. Hemagglutinating activity of protein extracts from marine algae for different types of erythrocytes

Species	Rabbit					Chicken					Sheep				
	N	T	B	P	S	N	T	B	P	S	N	T	B	P	S
Rhodophyta															
Spyridia clavata	–	–	55.4	27.7	–	27.7	3.5	3.5	6.9	27.7	13.8	1.7	0.9	3.5	3.5
Heterodasya sertularioides	7.3	3.6	3.6	3.6	3.6	–	–	–	–	–	–	–	–	–	–
Bostrychia scorpioides	–	–	–	–	–	–	–	–	–	–	–	164.7	82.3	41.2	164.7
Chondria sp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Laurencia sp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Polysiphonia ferrulacea	–	40.2	–	40.2	40.2	–	–	–	–	–	–	–	–	–	–
Calliblepharis occidentalis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gracilaria cylindrica	–	–	–	–	–	–	–	–	–	–	–	29.4	29.4	29.4	14.7
G. foliifera	38.2	76.5	76.5	38.2	76.5	76.5	19.1	38.2	38.2	38.2	76.5	39.2	19.1	19.1	19.1
G. verrucosa	–	–	–	–	–	–	32.6	–	–	–	–	–	32.8	32.8	–
G. curtissiae	15.4	7.7	7.7	7.7	7.7	123.5	30.8	7.7	7.7	3.9	30.9	7.7	7.7	3.8	7.7
G. venezuelensis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Kallymenia westit	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chrysimenia halimenioides	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Agardhiella ramosissima	–	8.1	8.1	4.1	4.1	–	–	–	–	–	16.3	16.3	4.1	8.1	8.1
Halymenia gelinaria	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H. pseudofloresia	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Galaxaura oblongata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G. obtusata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chlorophyta															
Acetabularia calyculus	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Anadyomene stellata	–	8.4	4.2	2.1	2.1	–	–	–	–	–	1078.0	539.2	539.2	539.2	539.2
Ulvaria oxysperma	–	–	–	–	–	–	–	–	–	–	–	46.8	46.8	46.8	93.6
Caulerpa cupressoides	95.6	47.8	47.8	47.8	47.8	–	–	382.3	–	–	–	–	–	–	–
Cladophoropsis membranacea	–	253.3	470.6	470.6	470.6	–	–	–	–	–	941.2	235.3	235.3	235.3	235.3
Phaeophyta															
Dictyopteris delicatula	–	118.8	–	118.8	–	–	–	–	–	–	–	–	–	–	–
D. justi	–	20.1	–	–	–	–	–	–	–	–	–	59.3	29.7	29.7	59.3
Dictyota mertensii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Zonaria tournefortii	–	–	–	–	–	–	–	–	–	–	–	–	–	89.7	–
Colpomenia sinuosa	–	–	–	–	–	–	–	–	–	–	–	–	105.9	105.9	–
Cyanophyta															
Lyngbya confervoides	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Minimum concentration (μg/ml) of protein which produced agglutination; (–) No detectable activity; N: non treated erythrocytes; T: trypsin treated erythrocytes; B: bromelain treated erythrocytes; P: papain treated erythrocytes; S: subtilisin treated erythrocytes.

Results

The results of the screening programme are summarized in (Table 1). Only *Caulerpa cupressoides* has been investigated previously for haemagglutinins. Sheep erythrocytes were the most suitable to detect the haemagglutinating activity followed by rabbit, human A, B, O and at lesser degree by chicken erythrocytes (Table 1; Figure 1).

Rhodophyta

Ten out of 19 species showed haemagglutinating activity against at least one type of erythrocyte. Protein extract of *Gracilaria foliifera* gave positive agglutination for all erythrocytes used. Extracts of *Gracilaria cylindrica* and *Polysiphonia ferrulacea* agglutinated only sheep and rabbit enzyme-treated erythrocytes, respectively. The protein extract of *Calliblepharis occidentalis* exhibited human blood-group A specificity while *Gracilaria curtissiae* appeared to

Table 1. (continued)

Species	A					B					O				
	N	T	B	P	S	N	T	B	P	S	N	T	B	P	S
Rhodophyta															
Spyridia clavata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Heterodasya sertularioides	–	–	6.8	17.8	–	–	116.7	7.3	58.3	58.3	58.3	58.3	14.6	29.2	29.2
Bostrychia scorpioides	82.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chondria sp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Laurencia sp	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Polysiphonia ferrulacea	–	–	–	–		–	–	–	–	–	–	–	–	–	–
Calliblepharis occidentalis	–	–	139.2	139.2	139.2	–	–	–	–	–	–	–	–	–	–
Gracilaria cylindrica	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G. foliifera	–	–	305.9	305.9	–	–	–	618.8	–	–	–	–	152.9	611.2	152.9
G. verrucosa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G. curtissiae	247.1	123.5	30.9	30.9	247.1	–	–	–	–	–	123.5	123.5	61.8	123.5	30.9
G. venezuelensis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Kallymenia westit	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chrysimenia halimenioides	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Agardhiella ramosissima	–	–	–	–	–	–	65.1	16.3	–	65.2	16.3	–	–	–	–
Halymenia gelinaria	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
H. pseudofloresia	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Galaxaura oblongata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
G. obtusata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Chlorophyta															
Acetabularia calyculus	–	–	108.8	–	–	–	–	108.8	–	–	–	–	–	–	–
Anadyomene stellata	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ulvaria oxysperma	–	–	–	–	–	46.8	46.8	23.4	93.6	46.8	–	–	–	–	–
Caulerpa cupressoides	191.2	47.8	47.8	191.2	47.8	95.6	23.9	3.0	11.9	23.9	191.3	47.8	47.8	191.2	47.8
Cladophoropsis membranacea	–	941.2	470.6	941.2	–	–	–	941.2	470.6	–	–	–	–	–	–
Phaeophyta															
Dictyopteris delicatula	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D. justi	59.3	59.3	118.6	–	–	–	–	–	–	–	–	–	–	–	–
Dictyota mertensii	–	–	40.2	–	–	–	–	20.1	–	–	–	–	20.1	–	–
Zonaria tournefortii	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Colpomenia sinuosa	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Cyanophyta															
Lyngbya confervoides	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Minimum concentration (μg/ml) of protein which produced agglutination; (–) No detectable activity; N: non treated erythrocytes; T: trypsin treated erythrocytes; B: bromelain treated erythrocytes; P: papain treated erythrocytes; S: subtilisin treated erythrocytes.

have the unusual anti A + H blood group specificity. The minimum amount of protein required to produced agglutination was observed in *Spyridia clavata* against sheep enzyme-treated erythrocytes. The protein extracts agglutinated all enzyme-treated erythrocytes more strongly than the native cells.

Chlorophyta

All protein extracts produced by the green algae agglutinated at least one kind of erythrocytes. The strongest activity was observed with *Anadyomene stellata* using rabbit enzyme-treated erythrocytes, although it failed to agglutinate human blood group erythrocytes. The protein extract of *Ulvaria oxysperma* exhibited specific haemagglutinating activity with erythrocytes carrying the B antigen; *Acetabularia calyculus* extract agglutinated only human A and B erythrocytes treated with bromelain at the same level. The protein extracts required a lower protein concentration to agglutinate enzyme-treated than untreated erythrocytes.

Phaeophyta

Protein extracts from all but one brown alga exhibited agglutinating activity against at least one type of erythrocyte. The exception was *Dictyopteris delicatula* which failed to agglutinate all types of cells tested. The extract of the closely related species *Dictyopteris justii* did, however, agglutinate rabbit and sheep enzyme-treated erythrocytes and was showing to be human blood group A specific. The extracts with positive agglutination towards enzyme-treated erythrocytes failed to agglutinated all the types of native erythrocyte.

Cyanophyta

The only one species tested, *Lyngbya confervoides*, failed to exhibit agglutination activity with all types of erythrocyte.

Haemagglutination inhibition studies

The five extracts showing the strongest haemagglutination titres were used for sugar inhibition studies with several simple sugars and glycoproteins (Table 2). Only the extract from *Caulerpa cupressoides* showed inhibition by simple sugars; all the glycoproteins failed to inhibit the haemagglutinating activity. Raffinose was the most potent inhibitory substance, followed by lactose and then equally by galactose and fructose. The agglutinating activity present in *Gracilaria foliifera, G. curtissieae, Agardhiella ramosissima* and *Heterodasya sertularidoides* was inhibited only by glycoproteins. Porcine stomach mucin and fetuin were the best substances inhibiting the protein extracts. Avidin and yeast mannan inhibited only the protein extracts of *Gracilaria foliifera* and *Agardhiella ramosissima*, respectively.

Discussion

Lectin activity in 63% of these previsouly unexamined species was detected, with positive results with at least against one of the different types of erythrocytes tested. From the results of this screening we observed that sheep and rabbit enzyme-treated erythrocytes were more sensitive to detect the haemagglutinating activity on the algal extracts examined. Similar observations with other Brazilian marine algae have been reported by Ainouz et al. (1992). Furthermore, animal erythrocytes have been reported to be more suitable for lectin detection in marine algae than human cells (Hori et al., 1988; Chiles & Bird, 1989; Fábregas et al., 1985; Hori et al., 1990; Dalton et al., 1995). Only four algae extracts tested showed specific agglutination towards human blood types. The unusual blood group specificity A + H, found in *Gracilaria curticiae* has been reported previously by Boyd et al. (1966), Rogers (1977), Hori et al. (1981) and Ainouz et al. (1992) in few species of marine algae. Remarkably, proteins extracts of the green alga *Ulvaria oxysperma* exhibited blood group B specificity, only found in the well stablished lectin from the red marine alga *Ptilota plumosa* (Rogers et al., 1977). Furthermore, blood group A specificity showed by extracts of the red alga *Calliblepharis occidentalis,* have been detected only in the green marine algae genus *Codium* (Loveless & Rogers, 1985; Rogers et al., 1994). Due to these interesting results, isolation and characterisation of these algal haemagglutinins are now under progress.

Haemagglutination inhibition studies of five algal species were carried out using simple sugars and glycoproteins. The lack of specificity for simple sugars by these algal preparations is in agreement with several works already reported in the literature (Hori et al., 1990; Rogers & Hori, 1993; Dalton et al., 1995) and appears to be a common feature of many algal lectins. However, lectins from the genus *Codium* have been reported to be inhibited by N-acetylgalactosamine. The red marine algae *P. plumosa* (Rogers et al., 1977) and *P. serrata* (Rogers et al., 1990) exhibited strong inhibition by galactose and their derivatives. The lectins from *Gracilaria tikvahiae* were inhibited by N-acetylneuraminic acid (Dalton et al., 1995). Only the activity present in *Caulerpa cupressoides* exhibited inhibition by the simple sugar galactose and the simple sugars containing galactose, lactose and raffinose. Interesting, the monosaccharide fructose was also inhibitory. The specificity for galactose and galactose-containing sugars in *C. cupressoides* are in agreement with the strongly preference for human erythrocytes carrying the B antigen (galactose as sugar determinant) than groups A and O.

As for most algal lectins, the glycoproteins showed to be good inhibitors of the haemagglutinating activity present in marine algae. Four out five glycoproteins tested inhibited haemagglutinating activity of the red alga *A. ramosissima*. Yeast mannan, a high mannose type glycoprotein was the most powerfull inhibitory substance. Interestingly, the simple sugar mannose did not inhibited the activity present in *A. ramosissima* protein extract. Porcine stomach mucin, a glycoprotein

Table 2. Inhibition of trypsin treated sheep erythrocyte agglutination activity

Substance Tested	Extract				
	G.f	A.r	C.c	G.c	H.s
Simple sugar					
D(+) Glucose	–	–	–	–	–
D(+) Galactose	–	–	12.5	–	–
Lactose	–	–	6.25	–	–
Salicine	–	–	–	–	–
D(+) Raffinose	–	–	1.56	–	–
L(+) Rhamnose	–	–	–	–	–
D(+) Manose	–	–	–	–	–
D(−) Arabinose	–	–	–	–	–
L(+) Arabinose	–	–	–	–	–
D(−) Cellobiose	–	–	–	–	–
D(+) Xilose	–	–	–	–	–
D(−) Fructose	–	–	12.5	–	–
D(+) Glucosamine	–	–	–	–	–
N-acetyl-D-glucosamine	–	–	–	–	–
N-acetyl-D-galactosamine	–	–	–	–	–
Glycoproteins					
Avidin	–	–	–	0.312	–
Egg white	–	1.250	–	–	–
Fetuin	0.625	1.250	–	0.625	1.250
Mannan from *Saccharomyces cerevisiae*	–	0.312	–	–	–
Mucin from Porcine Stomach	0.625	0.625	–	0.625	0.039

(−) No detectable inhibitory activity
G.f = *Gracilaria foliifera*, G.c = *Gracilaria curtissiae*, A.g = *Agardhiella ramosissima*, C.c = *Caulerpa cupressoides*, H.s = *Heterodasya sertularioides*.
Minimum concentration (mM or mg. ml^{-1} for simple sugars and glycoproteins, respectively) of the substance which produced inhibition.

with terminal GalNAc residue, as well having fucose and galactose as internal residues (Slomiany & Meyer, 1972), followed by egg white and fetuin, all bearing complex N-glycans structures, were two and four time less inhibitory. This recognition for more complex structures with differences in their glycan portion (high mannose type or hibrid N-glycans) has been reported for other algal lectins (Rogers & Fish, 1991).

The results published until now from several works on survey of seaweeds for haemagglutinins lead to the isolation and characterisation of these proteins. Indeed, marine algae lectin have been exhibited considerable interest due to their unique properties as a tool for many biological applications.

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