Journal of Industrial Microbiology & Biotechnology (2000) 24, 262–266

www.nature.com/jim

Agglutination of human and animal erythrocytes in marine unicellular algae

W-R Liao and R Huang

Institute of Oceanography, National Taiwan University, Taipei 100, Taiwan

Ethanol extracts of 12 marine unicellular algae were assayed for agglutinating activity against native and enzyme-treated human and animal erythrocytes. All of the algae assayed agglutinated at least one group of normal erythrocytes from humans. Notably, all algal extracts agglutinated erythrocytes of hemophilia patients arising from coagulation disorders. Meanwhile, all algae had a strong reaction with monkey erythrocytes, but to a lesser extent or not at all with sheep erythrocytes. Both trypsin and pronase improved the detection of most algal agglutinins and caused a drastic increase in hemagglutinating activity after treatment for 2 h or more. However, hemagglutinating activity decreased or disappeared completely when two extracts of different algal species were combined. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 262–266.

Keywords: agglutinating activity; marine unicellular algae; erythrocytes; enzymes

Introduction

Marine algae contain lectins or agglutinins, which are proteinaceous compounds and have specific binding characteristics with carbohydrates to produce unique biological activities [3,8]. Lectins can cause coagulation in human and animal erythrocytes, bacteria, yeast, blue-green algae and even mouse tumor cells [4,9,11,16]. They also stimulate mitogensis of human lymphocytes [12,15]. Although many marine algae have been assayed for agglutination of human and animal erythrocytes, those investigations were limited to macroalgae, ie seaweeds, in a few ecological areas [1,2,9,11,17]. Agglutinating activity varies greatly between algal species and the sources of erythrocytes [1,2,9]. Such activity can be induced markedly by modifying the extraction process, ie using enzyme treatments and additives [2,12–14,16]. To date, research on agglutination with human hemophiliac blood or other coagulation disorders by marine algae has not been reported. A few species of seaweeds have been purified with the biochemical characteristics of the lectins specified [5,11,17,18]. Rogers and Fish [17] reviewed the biochemical characteristics of lectins in various seaweeds and their hemagglutination with human erythrocytes. Yet lectins and their biochemical characteristics in unicellular algae remain unknown. Recently, we found agglutination of human and animal erythrocytes by the extracts of chrysophycean algae (Liao *et al*, unpublished data), revealing that lectins also exist in single-cell algae. Therefore, we examined more unicellular algae for hemagglutination for a better understanding of the biochemical characteristics of lectins. Additionally, we combined the extracts from different species and assayed their hemagglutination after combination.

Correspondence: R Huang, Institute of Oceanography, National Taiwan University, Taipei 100, Taiwan. E-mail: rang@ccms.ntu.edu.tw
Received 24 September 1999; accepted 6 January 2000

Materials and methods

Algal cultures

Twelve species of marine unicellular algae belonging to Cyanophyceae, Chlorophyceae, Bacillariophyceae, Dinophyceae, Prymnesiophyceae, Eustigmatophyceae and Rhodophyceae were used for the hemagglutination assays. Algal cells were grown xenically in unialgal cultures in an f/2 enriched seawater medium [10] at 25°C and 15–17 W m^{-2} irradiance of L:D = 12:12 before assaying. The algal cells used in the assay were in exponential phase (3–5 days after inoculation in the enriched medium).

Preparation of algal materials

Cultures (2–3-litre) of algae were filtered through Whatman glass filters (GF/C) at low-pressure vacuum, ie 1/2 atm, 2–3 drops of Milli Q water (previously distilled) were added to the filter to wash out any salt remaining on the cells and filter. Cells on the filter membrane were then freeze-dried at –20°C for 48 h. Dried cells on the filter were scraped down as pellets and weighed. Algal pellets of approximately 0.2 g immersed in liquid nitrogen were ground into powder and then kept at –20°C until used.

Algal extraction

The extraction procedure followed that of Kawakubo *et al* [14]. Ethanol (20%) was added to the algal powder (1:10, w:v), and the mixture was stirred overnight at 4°C. After centrifugation, the supernatant mixture was collected for use in the agglutination assay.

Preparation of erythrocytes

The agglutinating activity of algal extracts was assayed using fresh human and animal erythrocytes. Some of the human erythrocytes were normal A, B, O and AB groups, and others were coagulation disorder (hemophilia) assigned to failure in activating thrombin within the prothrombin time (PT) or the activated partial thromboplastin time (APTT). The disorders arise from the lack of factors VII,

VIII and IX in the blood of patients. Human blood was obtained from the Union Clinical Laboratory, Taipei, while monkey and sheep erythrocytes were purchased from Accurate Chemical & Scientific Corporation, New York. All of the erythrocytes were concentrated by centrifugation in a 50 mM phosphate buffer (pH 7.2) containing 0.1 M sodium chloride [13]. This phosphate-buffered saline (PBS) was used to dilute the concentrated erythrocytes to 2% of their original volume. The erythrocytes were then used directly (native) in the assay for agglutination or treated with 2% trypsin or 0.5% pronase in PBS at 37°C for 2 h (enzyme-treated) before using them in assays.

Hemagglutinating activity assay

Aliquots of algal extracts from serial two-fold dilutions were added separately to 96-well micro-V-plates. An equal volume of erythrocyte suspension was added to each well, mixed with extracts at room temperature and allowed to stand for 2 h. Hemagglutination was examined and its activity, which was a reciprocal of the highest two-fold dilution exhibiting positive agglutination, was recorded. The control was the aqueous ethanol without algal extracts. For each species, the assay was carried out in duplicate samples. Activity was determined with an error of $\pm 2^1$ titers.

Agglutinating activity of the combined extracts

Equal volumes of algal extracts from two algal species were mixed together and shaken. The mixture was placed in wells for the assay of hemagglutination as described above.

Inhibition test with sugars

Sugar (50 μl of 0.5 M) in PBS was mixed thoroughly with an equal volume of algal extract and kept at room temperature for 30 min. The extracts were assayed again against native erythrocytes of the human O group to make sure of their agglutinating activity prior to mixing. Then 50 μl of the mixture was added to a well of a micro-V-plate, mixed with an equal volume of 2% type O erythrocytes. Agglutination of erythrocytes was examined after 2 h. Sugars used in the test were the monosaccharides, D-glucose, D(+)-galacotse, D(+)-mannose, methy-α-D-glucoside, methyl-α-D-mannoside and the oligosaccharides, sucrose, lactose and raffinose.

Results

Agglutination of human erythrocytes

Agglutination of human erythrocytes by marine algae varies greatly among algal species and erythrocytes assayed. All of the algae assayed agglutinated at least one group of normal erythrocytes, with maximum activity reaching $2^{4–25}$ titers (Table 1). Meanwhile, almost all species agglutinated strongly native O and AB erythrocytes, but to a lesser extent for A erythrocytes. The blue-green alga *Synechococcus* sp and the green alga *Ankistrodesmus* sp displayed extremely strong affinity for O erythrocytes, ie 2^{24}–2^{25} titers, followed by *Hymenomonas* sp and *Pavlova salina*, reaching $2^{12–13}$ titers. Four species were non-specific for human blood cells.

Table 1 Hemagglutinating activities of marine unicellular algae against native and trypsin-treated human erythrocytes; the activity is expressed as titer

Algal species	Native				Trypsin			
	A	B	O	AB	A	B	O	AB
Cynophyceae								
Synechococcus sp	0	2^2	2^{24}	2^2	2^1	2^3	2^{24}	2^7
Chlorophyceae								
Ankistrodesmus sp	0	0	2^{25}	0	2^1	2^2	2^{12}	2^1
Chlorella ellipoidea	0	0	2^3	2^5	2^2	2^1	2^5	2^6
Dinophyceae								
Gyrodinium instriatum	2^4	2^1	2^4	2^2	2^4	2^4	2^4	2^3
Prorocentrum minimum	2^3	2^1	2^4	2^3	2^3	2^3	2^3	2^2
Prymnesiophyceae								
Hymenomonas sp	2^3	2^5	2^{13}	2^6	0	2^6	2^{14}	2^7
Pavlova salina	2^3	2^9	2^{12}	2^5	2^{12}	2^{12}	2^{12}	2^{10}
Rhodophyceae								
Porphyridium sp	2^5	2^4	0	2^5	2^{12}	2^7	2^7	2^8

Agglutination of hemophilia blood

With the blood of hemophilia patients, all of the algae assayed agglutinated APTT type erythrocytes, while six of them reacted positively with PT type erythrocytes (Table 2). *P. salina* demonstrated the strongest activity (2^{20} titers) against PT erythrocytes, while *Synechococcus* sp was highly active towards both PT and APTT type erythrocytes.

Agglutination of animal erythrocytes

Differential preferences for animal erythrocytes were also distinct in unicellular algae. Nearly all of the algae assayed reacted strongly with native erythrocytes from monkey, but not from sheep erythrocytes (Table 3). Monkey erythrocytes are more susceptible to algal lectins than sheep erythrocytes in agglutination.

Table 2 Hemagglutinating activities of marine unicellular algae against native and trypsin-treated human coagulation disorder erythrocytes; the activity is expressed as titer

Algal species	Native		Trypsin	
	PT	APTT	PT	APTT
Cynophyceae				
Synechococcus sp	2^{12}	2^{12}	2^{12}	2^{14}
Chlorophyceae				
Ankistrodesmus sp	0	2^4	2^1	2^5
Chlorella ellipoidea	0	2^{12}	2^1	2^{13}
Dinophyceae				
Gyrodinium instriatum	2^1	2^1	2^3	2^3
Prorocentrum minimum	2^1	2^1	2^1	2^1
Prymnesiophyceae				
Hymenomonas sp	2^{12}	2^5	2^1	2^5
Pavlova salina	2^{20}	2^1	2^{14}	2^4
Rhodophyceae				
Porphyridium sp	2^1	2^1	2^2	2^3

Table 3 Hemagglutinating activities of marine unicellular algae against animal erythrocytes; N: native, T: trypsin-treated, P: pronase-treated and the activity is expressed as titer

Algal species	Sheep			Monkey		
	N	T	P	N	T	P
Cynophyceae						
Synechococcus sp	0	2^6	2^6	2^{10}	2^{18}	2^{17}
Chlorophyceae						
Ankistrodesmus sp	0	2^{11}	2^{10}	2^{11}	2^{17}	2^{18}
Chlorella ellipoidea	0	2^6	2^7	2^{11}	2^{19}	2^{18}
Dinophyceae						
Gyrodinium instriatum	0	0	2^3	0	2^1	2^5
Prorocentrum minimum	0	0	2^3	0	0	2^8
Prymnesiophyceae						
Hymenomonas sp	0	0	2^{11}	2^4	2^{13}	2^{17}
Pavlova salina	0	0	2^2	2^8	2^{17}	2^{14}
Rhodophyceae						
Porphyridium sp	2^2	2^3	2^{10}	2^7	2^{13}	2^{14}

Agglutination with enzyme-treated erythrocytes

Pretreatment of erythrocytes with trypsin or pronase caused an increase of hemagglutinating activity in most of the assays, particularly in *P. salina* and *Porphyridium* sp for A and PT erythrocytes (Table 1, Figure 1). Meanwhile more species gave positive hemagglutination reactions after treatment with pronase than with trypsin. Each enzyme also inhibited markedly algal hemagglutination, eg *Ankistrodesmus* sp for O erythrocytes and *Hymenomonas* sp and *P. salina* for PT erythrocytes. The enzyme-induced activity peaks after treatment for 2 h or more (Figure 1). With animal erythrocytes, trypsin and pronase also improved detection, but the latter was superior to the former (Table 3).

Hemagglutination of the combined algal extracts

Agglutinating activities of the combined extracts varied with the species used. However, compared with individual species, the combined agglutinating activities were strongly inhibited in general (Table 4). The extremely active extracts of *I. galbana* and *P. salina* (2^{20} titer) against native PT erythrocytes (Liao *et al*, unpublished) became inactive after combination. Similar results of abolished activity occurred in most of the combinations. In contrast, the combination of *S. costatum* with *I. galbana* resulted in a drastic increase of agglutination against sheep erythrocytes. Meanwhile, all of the combined extracts retained high activity against APTT erythrocytes.

Inhibition of agglutination

Both mono- and oligosaccharides inhibited algal extracts in agglutinating erythrocytes (Table 5). However, the inhibition patterns of sugars varied with the algae tested. It

Table 4 Agglutination of the combined extracts of two marine algae against native erythrocytes; the activity is expressed as titer. The numbers in parentheses indicate the individual activities of two corresponding species from Liao *et al* (unpublished data)

Pairs of algal species	Erythrocyte			
	B	PT	APTT	Sheep
Skeletonema costatum	$(0,2^6)$	$(2^{14},2^{20})$	$(2^{12}, 2^7)$	(0,0)
Isochrysis galbana	2^8	2^{12}	2^6	2^8
S. costatum	$(0,2^3)$	$(2^{14},0)$	$(2^{12},2^{12})$	(0,0)
Cyclotella sp	0	0	2^5	0
S. costatum	$(0,2^4)$	$(2^{14},2^1)$	$(2^{12},2^1)$	$(0,2^2)$
Porphyridium sp	0	0	2^5	2^2
I. galbana	$(2^6,2^9)$	$(2^{20},2^{20})$	$(2^7,2^1)$	(0,0)
Pavlova salina	0	0	2^5	0

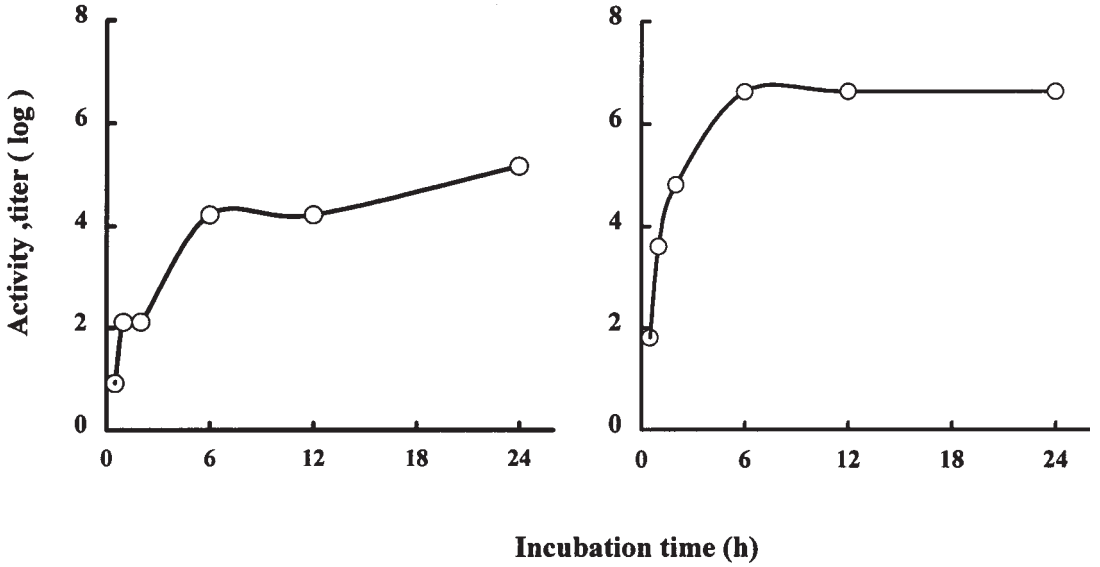

Figure 1 Hemagglutination by algal extracts of pronase-treated erythrocytes at various time intervals of incubation (left: *Hymenomonas* sp with O erythrocytes, right: *Porphyridium* sp with PT erythrocytes).

Table 5 Inhibition of algal agglutinating activity against native erythrocytes of human O group by sugars

Algal species	Monosaccharides					Oligosaccharides			
	1	2	3	4	5	6	7	8	9
Synechococcus sp	−	+	−	+	+	−	−	+	+
Ankistrodesmus sp	+	+	+	+	+	+	+	−	−
Gyrodinium instriatum	+	+	−	+	+	+	+	+	+
Prorocentrum minimum	+	+	+	−	+	+	+	+	−
Hymenomonas sp	−	−	+	−	−	+	+	−	−
Pavlova salina	−	+	+	−	−	−	+	−	−
Skeletonema costatum	+	+	+	+	+	+	+	+	−
Cyclotella sp	+	+	+	−	+	+	+	+	−

1: D-glucose; 2: D(+)-galactose; 3: D(+)-mannose; 4: methyl-α-D-glucoside; 5: methyl-α-D-mannoside; 6: sucrose; 7: lactose; 8: L-lactose; 9: raffinose; + = inhibition, − = noninhibition.

appeared that D(+)-galactose and lactose inhibited all of the algae tested except *Hymenomonas* sp and *Synechococcus* sp, respectively. *G. instriatum* and *S. costatum* remained active with D(+)-mannose and raffinose, respectively, but not with others.

Discussion

Like seaweeds, marine unicellular algae contain lectins that agglutinate human and animal erythrocytes to varying degrees. In light of their generally strong activities, marine unicellular algae are also a potent source of lectins for studies in biochemistry and medical applications.

Lectins of marine macroalgae have generally weak activity towards human erythrocytes, and are more specific for A and B than for O erythrocytes [2,4,5,9,16]. In this study, lectins in unicellular algae displayed comparatively high activities against human erythrocytes, particularly O and AB groups. Some of them have particularly interesting characteristics, eg extremely strong affinity for O erythrocytes by lectins from *Synechococcus* sp and *Ankistrodesmus* sp.

The generally strong activity against hemophilia erythrocytes in unicellular algae is most interesting since previous literature has rarely reported similar agglutination by marine macroalgae. These, together with our preliminary findings with chrysophyte cells (Liao *et al*, unpublished data) reveal that lectins which coagulate the anomalous blood, are widely distributed in marine unicellular algae.

Many authors used erythrocytes from sheep and other animals to assay hemagglutinins in seaweeds; they found a range of affinities with the algal species used [1,5,9,12,14]. In our study, most algae reacted strongly against monkey erythrocytes, but to a lesser extent or not at all with sheep erythrocytes.

Pretreatment of erythrocytes with enzymes, such as papain, neuraminidase, albumin, trypsin and pronase, can improve markedly the detection of lectins in seaweeds [2,16,17]. Such enzyme-dependent lectins are common in the algae assayed, however, the induced activity varies with the algal species. Meanwhile, treatment with pronase rather than trypsin could enhance all algal extracts to agglutinate both sheep and monkey erythrocytes. However, hemagglutination inhibition also occurred after enzyme treatment, particularly with human erythrocytes. Such enzyme inhibition has rarely been mentioned in the literature.

Lectins of seaweeds are generally characterized by glycoproteins, with or without binding to monosaccharides, and a requirement for divalent cations [17,18]. They specifically recognize and bind the carbohydrates in glycoproteins. Thus the sugar moiety of glycoproteins is the key in inhibiting algal hemagglutination. Many glycoproteins, glycopeptides and monosaccharides or simple sugars, eg D-glucose, D-mannose, D-galactose, D-fucose, *N*-acetylgalactosamine and *N*-acetylglucosamine, inhibit specifically lectins of various seaweeds in hemagglutination [5,11,17,18]. Our study also demonstrated inhibition by monosaccharides and oligosaccharides of the agglutinating activity of marine microalgae. In view of the photosynthetic storage food in algal cells, members of Chrysophyceae, eg the diatoms *S. costatum* and *Cyclotella* sp and the flagellates *I. galbana* and *P. salina*, have abundant chrysolaminaran, a polysaccharide predominated by glucose, and considerable amounts of galactose, mannose, fructose and fucose [6,7]. Thus the strong hemagglutination inhibition in the combined extracts of this study probably arose largely if not entirely from the binding (primary and/or secondary) of one or more of the above or related simple sugars to the lectins within two algal species. We do not clearly know their interactions and binding processes. It appears that different algal combinations exhibited somewhat different patterns of agglutination against the erythrocytes assayed, implying a variety of cellular carbohydrates (both internal and external) between species and among taxonomic groups. Blunden *et al* [2] and Fabregas *et al* [9] revealed that hemagglutinins and their activities in marine algae are taxonomically significant. Others reported agglutination of blue-green algae by seaweed extract [11]. There remains much work to explore the biochemical characteristics of lectins in unicellular algae, particularly inter- or intraspecies reactions of algal cells with extracellular substances, with reproduction as well as symbiosis of bacteria or viruses on algal cells [3,8].

Acknowledgements

The authors gratefully acknowledge Shien-Chi Huang of the Union Clinical Laboratory (UCL), Taipei for providing human blood samples and Heui-Mei Su of the Tungkang Fisheries Institute, Taiwan for providing algal cultures.

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