A COMPARATIVE STUDY OF ANIMAL ERYTHROCYTE AGGLUTININS FROM MARINE ALGAE

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Abstract—1. Fifteen marine algal species were analyzed for agglutinins to rabbit, sheep and human A, B and O blood group erythrocytes.

2. Protein extracts from all marine algae agglutinated rabbit erythrocytes, whereas twelve and five extracts agglutinated sheep and human erythrocytes, respectively.

3. The highest agglutination titers were consistently observed with rabbit erythrocytes.

4. *Dictyota dichotoma* strongly agglutinated human B blood group erythrocytes relative to A and O group erythrocytes.

5. Agglutination titer of rabbit erythrocytes by six algal extracts was not inhibited by mono- or polysaccharides, yet was reduced by glycoproteins.

INTRODUCTION

Agglutinins are heat-sensitive proteins or glycoproteins which can agglutinate a variety of foreign particles such as bacteria and vertebrate erythrocytes (Ratcliffe *et al.*, 1985). In most instances agglutination is due to the protein's polyvalent nature and affinity for specific mono- or oligosaccharides. These proteins are very similar to higher plant lectins, yet for the most part their carbohydrate-binding specificity is unknown. Although the role of agglutinins and lectins is unclear at present, it is thought that the former plays a role in invertebrate defense reactions by functioning as opsonins (Renwrantz and Stahmer, 1983) or acting as general antibacterial agents (Ratcliffe *et al.*, 1985). They have also been documented in a wide range of non-immunological functions such as mediating symbiosis (Ho and Malek-Hedayat, 1988) and promoting sponge cell reaggregation (Henkart *et al.*, 1973).

Agglutinins are widely distributed throughout higher plants (Goldstein and Hayes, 1978; Etzler, 1986) and vertebrates (Barondes, 1986). In comparison little information is available concerning their distribution in marine plants. A British survey of over 100 species of seaweed extracts revealed that only 19 species agglutinated human erythrocytes (Blunden *et al.*, 1975). One species, *Ptilota plumosa*, was highly active and specific for human B blood group erythrocytes. Marine algae collected from coastal Japan have also been the focus of study for agglutinins against animal erythrocytes (Hori *et al.*, 1981). In this study approximately 53 species of green, red and brown algae were examined; fourteen species agglutinated rabbit erythrocytes and 9 of these species were active against human erythrocytes. Recently, Hori *et al.* (1988) have extended their studies to the use of pronase-treated sheep blood cells. Using this approach 27 of 31 algal species studied caused agglutination of sheep erythrocytes.

Many of these earlier surveys examined total cellular extracts. Such preparations may contain soluble metabolites which can lead to variability and erroneous results. For example, polyphenols and monosaccharides can either cause nonspecfic agglutination or actually inhibit endogenous agglutinins, respectively. Thus, we have initiated a comparative study for the presence of agglutinins to rabbit, sheep and human erythrocytes using partially purified soluble proteins isolated from fifteen different marine algal species. The algae were collected along southeastern Florida coastal waters and are distributed among seven orders of the three major divisions Chlorophyta, Phaeophyta and Rhodophyta. The sugar-binding specificity of six algal agglutinins was also examined.

MATERIALS AND METHODS

Materials

Concanavalin A (Type IV), affinity chromatography material and most chemicals were obtained from Sigma Chemical Co. Tissue culture glassware and microtiter plates were purchased from Corning. Rabbit and sheep erythrocytes were obtained in a citrated solution from Colorado Serum Co. Human erythrocytes were furnished by Central Florida Blood Bank (Orlando, FL). Phosphate buffered saline (PBS) was from Gibco.

Collection of algal species

Marine algae were collected during July and August, 1988. *Halymenia floresia*, *Halymenia agardhii*, *Sargassum filipendula*, *Spatoglossum schroederi* and *Padina vickersiae* were collected off N. Hutchinson Island, Fort Pierce, FL. *Caulerpa sertularioides* and *Acanthophora spicifera* were collected along the Indian River in Fort Pierce, FL. *Codium taylori* and *Eucheuma nudum* were obtained by scuba off Key West, FL, whereas the *Dictyota dichotoma* was collected at Spanish Harbor Key, FL. *Caulerpa paspaloides* was gathered along Lake Surprise, Key Largo, FL. The remaining algae were obtained from culture collections located at Harbor Branch Oceanographic Institution and grown under

nitrogen sufficient outdoor culture conditions as described by Bird *et al.* (1982). The taxonomic reference for identification of algal species was Taylor (1972) or Dawes (1974).

Preparation of soluble protein extracts

All procedures were conducted at 4°C. The algae were harvested, cleaned and rinsed with distilled water (dH_2O). Algae (300 g) were homogenized in a Waring blender (1 min) containing approximately 100 ml of TBS (10 mM Tris, 150 mM NaCl, pH 7.4), immediately frozen and lyophilized. The dried algal material was then resuspended in 20 volumes (v/w) of TBS and stirred for 18 hr. Unbroken cells were removed by centrifugation at 10,000 × *g* (20 min). The supernatant was then filtred under vacuum (Whatman #4) and solid ammonium sulfate was added to 20% saturation. This mixture was stirred for 4 hr and then centrifuged at 10,000 × *g* (20 min). The supernatant was recovered and ammonium sulfate was added to yield a 75% saturated solution. After 16 hr, the proteins were collected by centrifugation (10,000 × *g*, 20 min) and resuspended in 20 ml of TBS. The resuspended material was extensively dialysed (Spectrapor, cutoff 6000–8000 daltons) against three changes of PBS (18 l × 16 hr). Insoluble material was removed by vacuum filtration (Gelman GF/A) and the protein solutions stored at −20°C. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Assay for hemagglutination activity

Approximately 4 ml of serum containing erythrocytes was washed three to five times with 3 volumes of PBS. The supernatant from the final wash was decanted and a 2% (v/v) erythrocyte cell suspension was prepared in PBS. Approximately 40 μl of PBS was added to each well of a 96-well round bottom microtiter plate. An equal volume of algal protein extract was then prepared by serial two-fold dilution. Approximately 40 μl of the 2% erythrocyte suspension was added to each well, plates were mixed on an orbital shaker and incubated at room temperature (R.T.). After 2 hr, each well was examined for agglutination. The activity is expressed as the minimum protein concentration which produces agglutination. Controls designed to measure agglutinating and nonagglutinating activity were Concanavalin A, Type IV and PBS, respectively. Concanavalin A typically agglutinated rabbit and sheep erythrocytes at a minimum concentration of approximately 1.0 μg/ml and 7.8 μg/ml for human erythrocytes.

Hemagglutination inhibition assay

The monosaccharides used were D-mannose, D-glucose, D-galactose, L-fucose, *N*-acetylglucosamine, *N*-acetylneuraminic acid, D-glucuronic, D-mannuronic and D-galacturonic acids. Polysaccharides were alginate, maltose, κ-carrageenan and agarose, whereas the glycoproteins were fetuin, α_1-acid glycoprotein and lactoferrin. Agglutination was assayed as described above, yet before the addition of erythrocytes 40 μl of PBS or PBS containing 75 mM monosaccharide or 2 mg/ml polysaccharide was added to each well. In contrast, the glycoproteins were tested by adding 40 μl of algal protein extract (titer of 1:64) to each well. An equal volume of PBS or PBS containing the indicated glycoprotein at 8 mg/ml was then added and diluted serially two-fold. All plates were mixed and incubated at R.T. After 15 min, approximately 40 μl of the 2% rabbit erythrocyte suspension was then added. Within 2 hr, the wells were examined for agglutination. Rabbit erythrocytes were used in these studies because each algal extract produced strong agglutination of these erythrocytes.

Affinity chromatography

The sugars employed for affinity chromatography were D-mannose, L-fucose and *N*-acetylglucosamine coupled to agarose. The polysaccharides were agarose, κ-carrageenan and alginate beads; the later two types of beads were prepared by the method of Brodelius (1984). Chromatography of algal extracts was conducted at 4°C using approximately 1 ml of affinity matrix in 0.5 × 5 cm glass pipettes. The columns were equilibrated with PBS and 0.5 ml of algal extracts (600 μg protein/ml) was applied at a flow rate of 0.5 ml/min. The flow through was collected and reapplied onto the column a total of four times. Unbound material was collected by washing the column with 4 volumes of PBS, concentrated and assayed for agglutination. The columns were regenerated by successive washing with: 20 mM Tris, 0.5 M NaCl, pH 4.5; 20 mM Tris, 0.5 M NaCl, pH 8.0; PBS.

RESULTS

The results of screening marine algae for the presence of animal erythrocyte agglutinins are summarized in Table 1. Seven algal species from the Rhodophyta and four species each from the Chlorophyta and Phaeophyta divisions were studied. Protein extracts from all algal species agglutinated rabbit erythrocytes. Comparatively, agglutination of rabbit blood cells was the strongest among the five animal erythrocytes tested. Ten algal extracts were active against rabbit erythrocytes even at concentrations as low as 90–100 ng protein/ml. The red algal, *Acanthophora spicifera* strongly agglutinated rabbit erythrocytes, yet was not active against sheep or human, A, B or O blood groups.

For every algal extract examined, the concentration of protein required to agglutinate sheep erythrocytes was substantially greater than that observed for rabbit erythrocytes. Three species, *Halymenia floresia*, *H. agardhii* and *Gracilaria verrucosa* strain G-16S were active only at protein concentrations equal to or greater than 1 mg/ml. The largest difference in activity between rabbit and sheep erythrocytes was seen with both algae from the genus *Halymenia*.

Only seven of the fifteen marine species examined agglutinated human blood group erythrocytes. Among the active algal species there was little specificity towards a given human blood group. Except for *Dictyota dichotoma*, all species were equally active toward both human A and B blood groups. Although *D. dichotoma* agglutinated human A group erythrocytes, it reacted much more strongly with group B erythrocytes. *Caulerpa sertularioides* strongly agglutinated all three human blood groups.

The results of experiments designed to compare the sugar-binding specificity of six algal extracts distributed among the three marine plant divisions are presented in Table 2. No mono- or polysaccharides tested at concentrations of approximately 20 mM and 0.5 mg/ml, respectively, inhibited agglutination of rabbit erythrocytes. D-Mannose and D-galactose reduced the agglutination titer of *H. agardhii* and *Codium taylori*; however, the degree of inhibition was not consistent. Even at monosaccharide concentrations in excess of 75 mM, no inhibition was observed (data not shown). The agglutination titer produced by Concanavalin A was routinely inhibited by D-mannose and *N*-acetylglucosamine. On the other hand, activity from each species was reduced by the addition of various glycoproteins (Table 2).

Table 1. Hemagglutination activity of marine algae

	Erythrocytes				
			Human		
Species	Rabbit	Sheep	A	B	O
Rhodophyta					
Ceramiales					
Acanthophora spicifera	0.1	NA	NA	NA	NA
Gigartinales					
Agarhiella ramosissima	204	410	NA	NA	NA
Eucheuma nudum	90*	1.4	1.9	1.9	NA
Gracilaria tikvahiae G-3	3.2	51.2	61.5	61.5	NA
Gracilaria verrucosa G-16S	1.6	1639	15.4	61.5	NA
Cryptonemiales					
Halymenia agardhii	90*	2000	NA	NA	NA
Halymenia floresia	90*	1000	NA	NA	NA
Phaeophyta					
Dictyotales					
Dictyota dichotoma	90*	5.7	123	1.9	NA
Padina vickersiae	0.1	NR	NA	NA	NA
Spatoglossum schroederi	0.1	NR	15.3	30.7	NA
Fucales					
Sargassum filipendula	7.8	500	NA	NA	NA
Chlorophyta					
Siphonales					
Caulerpa paspaloides	90*	2.9	NA	NA	NA
Caulerpa sertularioides	90*	31	0.1	0.1	15.3
Codium taylori	90*	735	0.1	0.1	NA
Ulotrichales					
Ulva lactuca	1.6	102	NA	NA	NA

Protein extracts from marine algae were examined for agglutination of animal erythrocytes as described in Materials and Methods. The data are represented as the minimum concentration (μg/ml) of protein which produced agglutination.
*Minimum concentration of protein which produced agglutination is expressed as ng/ml.
NA: no detectable activity.
NR: activity was not reproducible.

Lactoferrin inhibited the activity of all marine algae tested with concentrations ranging from 7.8 to 62.5 μg protein/ml. Agglutination of rabbit erythrocytes by four marine species was inhibited by fetuin; the activity from *Ulva lactuca* and *D. dichotoma* was not affected. α_1-Acid glycoprotein and the nonglycosylated protein, carbonic anhydrase (data not shown), did not effectively inhibit activity of any algal extract.

Due to the lack of measurable inhibition by directly including mono- or polysaccharides in the hemagglutination assay, an alternative approach was employed to elucidate the sugar-binding specificity for five of the six extracts examined in Table 2. Protein extracts from marine algae were subjected to repeated affinity chromatography on a variety of mono- and polysaccharide resins. Following chromatography, the unbound material was examined for a reduction in rabbit erythrocyte agglutination titer. As seen in Table 3, there appeared to be little sugar-binding specificity for the saccharides examined. Of the five marine species analyzed, only activity from *H. agardhii* was reduced by approximately one-fourth following chromatography on L-fucose. The activity of Concanavalin A was reduced approximately two-thirds following chromatography on D-mannose or *N*-acetylglucosamine affinity columns.

Table 2. Inhibition of rabbit erythrocyte agglutination activity by carbohydrate related compounds

	Saccharide*		Glycoprotein		
Algae	Mono-	Poly-	Fetuin	αAG	Lactoferrin
U. lactuca	—	—	—	—	7.8
C. taylori	—	—	1000	—	62.5
C. paspaloides	—	—	2000	—	15.6
H. agardhii	—	—	250	—	15.6
E. nudum	—	—	1000	—	31.3
D. dichotoma	—	—	—	—	31.5
ConA†	D-Man, NAG	—	NT	NT	NT

Algal extracts were tested for inhibition of agglutination by glycoproteins, mono- and polysaccharides as described in Materials and Methods. The saccharide and glycoprotein data are represented by the carbohydrates which produced inhibition and as the minimum concentration (μg/ml) which resulted in inhibition, respectively.
Bars indicate no inhibition. α_1-Acid glycoprotein is abbreviated αAG.
*Saccharides tested are listed in the Materials and Methods section.
†Concanavalin A (ConA) was used as a positive control.
NT: not tested.

Table 3. Hemagglutination activity of algal extracts following carbohydrate affinity chromatography

	Affinity matrix						
Algae	Con	Fuc	Man	NAG	Agar	Algn	κCn
U. lactuca	2^{10}	2^{10}	2^{10}	2^{10}	2^{10}	2^{10}	2^{10}
C. taylori	2^{12}	2^{12}	2^{12}	2^{12}	2^{12}	2^{12}	2^{12}
C. paspaloides	2^{11}	2^{11}	2^{11}	2^{11}	2^{11}	2^{11}	2^{11}
H. agardhii	2^{12}	2^{9}	2^{12}	2^{12}	2^{12}	2^{12}	2^{12}
E. nudum	2^{11}	2^{11}	2^{11}	2^{11}	2^{11}	2^{11}	2^{11}
ConA	2^{6}	2^{5}	2^{2}	2^{2}	NT	NT	NT

Algal extracts were examined for reduction of rabbit erythrocyte agglutination titer following affinity chromatography as described in Materials and Methods. The activity is expressed as titer, the reciprocal of the highest dilution that produced positive agglutination (i.e., $1:1024 = 2^{10}$). Symbol key: Con (Control), Fuc (L-fucose), Man (D-mannose), NAG (*N*-acetylglucosamine), Agar (agarose), Algn (alginate) and κCn κ-carrageenan).

*Concanavalin A (ConA) was used as a positive control.

NT: not tested.

DISCUSSION

Every marine algae tested exhibited agglutination activity to at least one of the five different animal erythrocytes examined. In comparing the agglutination of the five erythrocytes all marine algae strongly agglutinated rabbit blood cells. Twelve and seven species of algae were active with respect to sheep and human A, B or O group erythrocytes, respectively. In general, the minimum concentration of protein from each extract needed to produce visual agglutination was lowest with rabbit erythrocytes. Similar observations with other marine algae have reported that rabbit erythrocytes are perhaps the most suitable cell type for initial screening purposes (Hori *et al.*, 1981; Fabregas *et al.*, 1985).

Among the seven algal extracts which were active towards human erythrocytes, little specificity was detected for either human A, B or O blood groups. Six of the algal species agglutinated both A and B blood groups equally; *C. sertularioides* also active with type O group. In fact, the minimum concentrations of protein producing agglutination was identical in practically every case. The only algae which demonstrated specificity towards a human blood group was *D. dichotoma*. Reaction with type B blood was much stronger than with type A; no agglutination of type O blood was observed. Interestingly, of the 150 algal species reported in the literature only three, *Bryopsis hypnoides*, *Laurencia undulata* and *Ptilota plumosa*, have been reported specific for human B blood group (Blunden *et al.*, 1975; Hori *et al.*, 1981).

Agglutination reactions of six algal species were examined for inhibition by simple sugars, polysaccharides and glycoproteins. Because the agglutination of animal cells by many higher plant lectins is inhibited by specific monosaccharides, we reasoned that agglutination of rabbit erythrocytes by algal agglutinins should be inhibited by a similar process. The majority of monosaccharides chosen for the inhibition analysis were those which constitute high mannose or complex type oligosaccharide chains *N*-glycosidically linked, as well as *O*-glycosidically linked (Baumann and Doyle, 1984). Although a limited number of monosaccharides were employed, no inhibition of agglutination activity was observed when the saccharides were assayed directly. Furthermore, no reduction of rabbit erythrocyte agglutination titer was observed following monosaccharide affinity chromatography of the algal extracts.

The lack of detecting sugar-binding specificity from these algal extracts may be the result of a more complex recognition process by the agglutinins, one which may involve oligosaccharides instead of single monosaccharides. Alternatively, multiple agglutinins each with unique sugar-binding specificity may be present in a single algal species. Multiple lectins from a single organism have been reported and analyzed by a variety of methods including cross-absorption with different cells (Pauley, 1974) and carbohydrate affinity chromatography (Bretting *et al.*, 1976). In reference to the later method, protein extract from *C. taylori* was subjected to sequential chromatography on L-fucose, D-mannose and *N*-acetylglucosamine resins, yet no reduction of agglutination titer was observed (data not shown).

With respect to the possible recognition of oligosaccharides by algal agglutinins, activity of six marine species was inhibited by the addition of glycoproteins, such as lactoferrin; four species were inhibited by fetuin. Because the non-glycosylated protein, carbonic anhydrase, did not affect activity and the observation that inhibition by the glycoproteins was selective (i.e. *U. lactuca* and *D. dichotoma* was not affected by fetuin), the reduction of agglutination activity would appear to be attributed to the associated oligosaccharide. Invertebrate lectins isolated from a variety of sources (i.e. *Axinella polypoides* and *Crassostrea gigas*) also appear to recognize the oligosaccharide portion of glycoproteins rather than monosaccharides (Yeaton, 1981). A recent study detailing the analysis of sugar-binding specificities of algal agglutinins was reported by Hori *et al.* (1986). These investigators reported that monosaccharides were basically ineffective as inhibitors of agglutination; however most hemagglutinins were strongly inhibited by many glycoproteins and glycopeptides, such as ovomucoid, yeast mannan and fetuin.

The results of this study suggest that agglutinins are widely distributed among many species of marine algae and may recognize complex carbohydrates associated with components of membrane proteins or

lipids. Obviously, these observations warrant further investigation to determine the exact oligosaccharide-binding specificity. Studies are currently in progress at purifying agglutinins from *Gracilaria* and ascertaining their specificity towards biologically relevant carbohydrates, as well as purified *N*- and *O*- glycosidically linked oligosaccharides.

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