魚 Diplodus sargus L.の精子に対する海藻エキスの凝集活性

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Agglutination Activity of Algal Extracts Against Spermatozoa of the Fish *Diplodus sargus* L.

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Spermatozoa from the fish *Diplodus sargus* L. were agglutinated by seaweed extracts. Three interaction types were observed: head to head, tail to tail and head to tail. There was not singular agglutination for each algal species tested.

Of all algal extracts, 16% of brown algae and 3% of red algae, showed agglutinating activity. In the brown algae, the maximum agglutinating activity was shown by *Fucus serratus* (1:256), whereas in the red algae the maximum agglutinating activity was shown by *Polyneura halliae* (1:32). None of the green algae assayed showed agglutinating activity.

Some algal extracts can recognize specific binding sites on the spermatozoa membrane. In the inhibition assay, four carbohydrates showed inhibition activity in the case of *Fucus spiralis*: 1-β-galactopyranoside (96.8% inhibition) and maltose, glucose and mannose (87.5% inhibition). The red alga *Polyneura hilliae* activity was totally inhibited by *N*-acetyl-glucosamine and *N*-acetyl-mannosamine, and another red alga *Gelidium cartilagineum* by *N*-acetyl-glucosamine.

It is supposed that marine algal agglutinins offer an important tool to study cell surface and can be used in the discrimination of fish spermatozoa.

Agglutinins are proteins or glycoproteins having specificities for carbohydrate structures, binding selectively to red blood cells and microorganisms. They are widely distributed in higher plants and invertebrates.[1-3]

Lectins agglutinate a wide spectrum of animals cells, mainly erythrocytes, although spermatozoa agglutination is little known. The first use of lectins and lectin-like substances in studying sperm surface properties was with soy bean agglutinin from a variety of strains, which interacted with bull spermatozoa to produce predominantly tail to tail agglutination.[4]

Later, interactions of lectins and lectin-like substances were studied with sperms of different animals, sperms of several species of clams against a vast number of plant and seed extracts to study agglutination[5]; human sperms from differents donors which displayed differential agglutinability with various lectins depending on the blood group (ABO) specificity[6] and mouse sperms[7] and rabbit and hamster sperms,[8] whose binding sites to different lectins were determined.

Marine algal agglutinins seem to be widely distributed.[9-16] Their agglutinating activity was studied against animal and human erythrocytes, but it is not known against fish spermatozoa.

We report here the agglutinating activity of 62 species of seaweeds against the fish *Diplodus sargus* spermatozoa.

Materials and Methods

Collection and Extraction of Algae

Marine algae were collected from different points of the N. W. Coast of Spain and were kept at −20°C until used. Specimens (10 g) of each algal species were thawed and washed with distilled water. Each specimens was minced in a mortar with quartz sand (1:1, w/w), which had been previously washed four times with 0.5 N HCl, ten times with distilled water, and then oven-dried at 180°C for 6 h. Negative results were obtained when this sand was used for hemagglutinating activity in a control test. The alga-sand mixture was homogenized with 10 m*l* of PBS (phosphate buffered saline, pH 7.2), and centrifuged at 1000× g for 20 min. The supernatant was removed, filtered through a 0.22 μm Millipore filter and kept at −20°C until testing shortly afterwards.

The algal nomenclature used follows that Parke and Dixon.[17]

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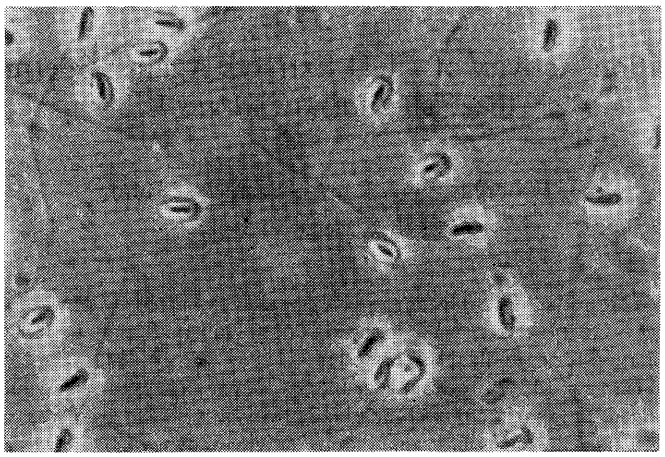

Fig. 1. *Diplodus sargus* spermatozoa (×1000 phase contrast).

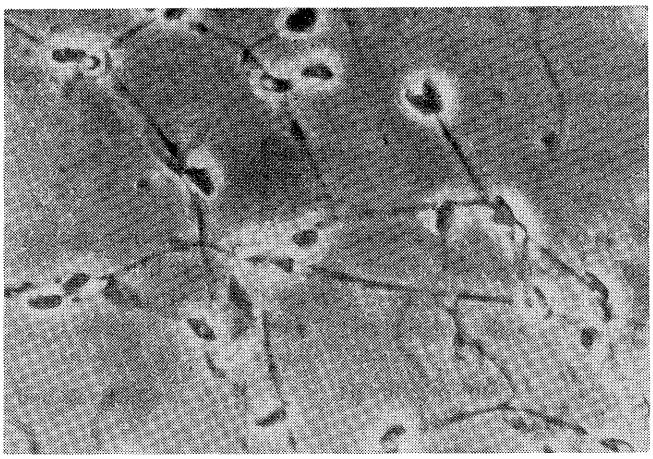

Fig. 2. Agglutinated *Diplodus sargus* spermatozoa (×1000 phase contrast).

Spermatozoa

Spermatozoa were obtained from a mature individual of *Diplodus sargus* L., by delicate ventral pressure, giving approximately 5 m*l* of semen. The harvesting was carried out into a sterile glass-vessel and was maintained at 4°C until testing. The microscope observation allows observing a head like bean (2–3 μm) and a long and motile flagellum (40–50 μm).

The spermatozoa were washed three times with seawater, which was previously filtered by 0.22 μm and, finally, a 1% spermatozoa suspension was prepared.

Assay for Agglutinating Activity

Tests of spermatozoa agglutination were carried out in excaved plates, with 20 μl of each algal extract and 20 μl of spermatozoa suspension. Previously, it was verified that less dilutions of spermatozoa suspension resulted in enough density for a suitable microscopic observation. If the agglutination was positive, a titration in the same plate was carried out, using two-fold dilutions of algal extracts in seawater. Incubation of the sperm suspension in seawater, giving no agglutination, was always performed as control.

The results of agglutination were expressed as titre, the highest two-fold dilution giving positive agglutination.

Inhibitory Effect of Saccharides on the Agglutinating Activity

For the measurement of sperm-agglutination inhibition by sugars, 19 kinds of them were dissolved in PBS at a concentration of 75 mM. As a control test, 20 μl of each sugar solution was added to 20 μl of spermatozoa suspension, giving a negative result. The inhibition tests were carried out making two-fold dilutions of each algal extract in seawater (50 μl) to which 50 μl of sugar solutions

Table 1. Agglutination activity of algal extracts against *Diplodus sargus* spermatozoa

	TITRE
BROWN SEAWEEDS	
ECTOCARPALES	
ECTOCARPACEAE	
Ectocarpus confervoides Le Jol.	—
Giffordia granulosa (Sm.) Hamel	1 : 2
CORYNOPHLAEACEAE	
Leathesia difformis (L.) Aresh.	
CHORDARIACEAE	
Mesogloia vermiculata (Sm.) S. F. Gray	—
SCYTOSIPHONACEAE	
Scytosiphon lomentaria (Lyngb.) Link	—
DESMARESTIALES	
DESMARESTIACEAE	
Desmarestia aculeata (L.) Lamour.	—
LAMINARIALES	
CHORDACEAE	
Chorda filum (L.) Stackh.	—
LAMINARIACEAE	
Laminaria saccharina (L.) Lamour.	—
Laminaria ochroleuca Pyl	—
Saccorhiza polyschides (Lightf.) Batt.	—
SPHACELARIALES	
STYPOCAULACEAE	
Halopteris scoparia (L.) Sauv.	—
CLADOSTEPHACEAE	
Cladostephus spongiosus (Huds.) C. Ag.	—
DICTYTALES	
DICTYOTACEAE	
Dictyopteris membranacea (Stackh.) Batt.	—
Dictyota dichotoma (Huds.) Lamour.	—
Taonia atomaria (Woodw.) J. Ag.	—
FUCALES	
FUCACEAE	
Ascophylum nodosum (L.) Le Jol.	1 : 2
Fucus serratus L.	1 : 256
Fucus spiralis L.	1 : 128
Fucus vesiculosus L.	1 : 64
Fucus ceranoides L.	1 : 8
Pelvetia canaliculata (L.) Donc et Thur	1 : 128
HYMANTALIACEAE	
Himanthalia elongata (L.) S. F. Gray	1 : 8
CYSTOSEIRACEAE	
Bifurcaria bifurcata Ross	—
Cystoseira baccata (Gmel.) Silva	1 : 4
Cystoseira foeniculacea (L.) Grev.	—
Cystoseira nodicaulis (With.) Roberts	—
Cystoseira tamarascifolia (Huds.) Papenf.	—
Halidrys siliquosa (L.) Lyngb.	1 : 64
RED SEAWEEDS	
BANGIOPHYCIDEAE	
BANGIALES	
BANGIACEAE	
Prophyra umbilicalis (L.) J. Agardh.	—
FLORIDOPHYCIDEAE	
CERAMIALES	
CERAMIACEAE	
Callithamnion tetragonum (With.) S. F. Gray	—
DASYACEAE	
Dasya ocellata (Grateloup.) Harv. in Hook	—
Heterosiphonia plumosa (J. Ellis) Batters	—
DELESSERIACEAE	
Cryptopleura ramosa (Huds.) Kylin ex New.	—
Polyneura hilliae (Grev.) Kylin	1 : 32
RHODOMELACEAE	
Chondria coerulescens (J. Agardh.) Falkenb.	—
Chondria tenuissima (Good. et Woods.) F. Schmitz	—
Laurencia pinnatifida (Huds.) Lamour.	—
Polysiphonia brodiaei (Dillwyn) Spreng	—
Polysiphonia lanosa (L.) Tandy	—
Pterosiphonia complanata (Clem.) Falkenb.	—
CRYPTONEMIALES	
DUMONTIACEAE	
Dilsea carnosa (Schm.) O. Kuntz	—
Dumontia incrassata (O. F. Mull) Lamour.	—
GIGARTINALES	
CYSTOCLONIACEAE	
Calliblepharis ciliata (Huds.) Kutz	—
Calliblepharis jubata (Good. et Woodw.) Kutz	—
GIGARTINACEAE	
Chondrus crispus Stackh.	—
Gigartina acicularis (Roth.) Lamour.	—
Gigartina pistillata (S. G. Gmelin) Starckh.	—
GRACILARIACEAE	
Gracilaria verrucosa (Huds.) Papenfuss.	—
GYMNOPHLAEACEAE	
Schyzimenia dubyi (Chauv. ex Duby) J. Agardh.	—
PLOCAMIACEAE	
Plocamium cartilagineum (L.) Dixon	
NEMALIALES	
GELIDIACEAE	
Gelidium pusillum (Stackh.) Le Jolis	—
Gelidium sesquipedale (Clem.) Thur et Born	—

Table 1. (Continued)

Gelidium cartilagineum Gaill	1:8	LOMENTARIACEAE	
BONNEMAISONIACEAE		*Lomentaria articulata* (Huds.)	
Asparagopsis armata (Harv.)	—	Lyngb.	—
Falkenbergia rufolanosa (Harv.)		GREEN SEAWEEDS	
F. Schmitz.	—	ULVALES	
Bonnemaisonia asparagoides		ULVACEAE	
(Woodw.) C. Agardh	—	*Enteromorpha sp.* Link in Nees	—
RHODYMENIALES		*Enteromorpha clathrata* (Roth.)	
CHAMPIACEAE		Grev.	—
Chylocladia verticillata (Lightf)		CODIALES	
Bliding	—	CODIACEAE	
Gastroclonium ovatum (Huds.)		*Codium tomentosum* Stackh.	—
Papenfuss.	—		

was added. This mixture was incubated at room temperature for 5 min and, finally, the same volume of spermatozoa suspension was added. A less titre was observed when there is inhibition effect. Per cent reduction in titers was calculated from the expression (A-B/A) times 100, where A is the titer of extract alone and B is the titer of extract with sugar.

Results and Discussion

In the agglutination tests we used fresh spermatozoa, although it is possible to use aldehyde-fixed sperms, but these probably have a reduced affinity for most lectins.[18]

In our case, the direct agglutination study in plates was more suitable, although it is possible to use microscope agglutination assay.[8] When a positive result was obtained, subsequent microscopic observation was carried out for estimating the agglutination types according to the classification of sperm agglutination by lectins: head to head, tail to tail, and head to tail.[8]

In *Diplodus sargus* sperm agglutination by algal extracts, the three interaction types were observed: head to head, tail to tail, and head to tail (see Figs. 1 and 2). Probably due to the use of crude algal extracts, a single type of agglutination for each algal species was not observed. However, most of the commonly used lectins appear to have a higher affinity for plasma membrane regions associated with the head rather than the flagellum.[18]

The sperm surface of *Diplodus sargus* probably presents specific binding sites for marine algal agglutinins. Of 62 extracts tested (Table 1) 19% of the extracts recognized the sperm surface: 16% of brown marine algae showed agglutinating activity, with a maximum in *Fucus serratus* (1:256); 3% of red marine algae reacted with spermatozoa, with a maximum in *Polyneura hilliae* (1:32). None of the green algae tested showed agglutinating activity against spermatozoa.

The high reactivity of brown marine algae can be due to their high content in polyphenolic substances, as it can be observed in the agglutination of the erythrocytes.[16,19] In general, this marked agglutinating activity is non-specific.

Some algal extracts present certain specificity, because they can recognize specific binding sites on the spermatozoon membrane. This can be deduced from the results of sugar inhibition.

In the case of *Fucus spiralis*, four carbohydrates showed inhibition (Table 2): 1-β-galactopyranoside (96.8% inhibition) and maltose, glucose and mannose with 87.5% of inhibition. The agglutinating activity of red algae *Polyneura hilliae* and *Gelidium cartilagineum* was totally inhibited by *N*-acetyl-glucosamine and *N*-acetyl-mannosamine, in the first case, and by *N*-acetyl-glucosamine in the second case.

We suppose that marine algal agglutinins offer an important tool to study cell surface. These characteristics are presented by plant lectins, which possess various affinities for particular carbohydrate residues and they are useful for investigating cell surfaces.[20]

As agglutinins specifically cause sperm agglutination, it is possible to identify different species.[8] Marine algal agglutinins can also be used in the discrimination of fish spermatozoa.

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Table 2. Inhibition of the agglutination by sugars and their derivates (75 mM)

	Lactose	myo-Inositol	*N*-Acetyl-D-Glucosamine	Maltose	Sorbitol	Sacarose	Glucose	Mannose	1-β-Galactopyranoside	Melibiose	α-methyl-D-Mannoside	Melezitose	Fructose	Glucosamine	Galactose	Adonitol	Trehalose	*N*-Acetyl-Mannosamine	Arabinose
Halidrys siliquosa	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ascophylum nodosum	—	—	—	—	87.5%	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bifurcaria bifurcata	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fucus ceranoides	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fucus serratus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fucus spiralis	—	—	—	87.5%	—	—	87.5%	87.5%	96.8%	—	—	—	—	—	—	—	—	—	—
Giffordia granulosa	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cystoseira baccata	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Himanthalia elongata	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Polyneura hilliae	—	—	100%	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Gelidium cartilagineum	—	—	100%	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Codium tomentosum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

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