Sulfated fucans from the egg jelly of sea urchins

Sulfated fucans from the egg jelly of the closely related sea urchins Strongylocentrotus droebachiensis and S. pallidus ensure species-specific fertilization*

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SUMMARY

Sulfated polysaccharides from the egg jelly are the molecules responsible for inducing the sperm acrosome reaction in sea urchins. This is an obligatory event for sperm binding to, and fusion with, the egg. The sulfated polysaccharides from sea urchins have simple, well-defined repeating structures and each species represents a particular pattern of sulfate substitution. Here, we examine the egg jellies of the sea urchin sibling species *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus*. Surprisingly, females of *S. droebachiensis* possess eggs containing one of two possible sulfated fucans, which differ in the extension of their 2-O-sulfation. Sulfated fucan I is mostly composed of a regular sequence of 4 residues, as follows: \([4-\alpha-L-Fucp-2(\text{OSO}_3)-1\rightarrow4-\alpha-L-Fucp-2(\text{OSO}_3)-1\rightarrow4-\alpha-L-Fucp-1\rightarrow4-\alpha-L-Fucp-1]_n\) whereas sulfated fucan II is a homopolymer of \([4-\alpha-L-Fucp-2(\text{OSO}_3)-1] \) units. Females of *S. pallidus* contain a single sulfated fucan with the following repetitive structure: \([3-\alpha-L-Fucp-2(\text{OSO}_3)-1\rightarrow3-\alpha-L-Fucp-2(\text{OSO}_3)-1\rightarrow3-\alpha-L-Fucp-4(\text{OSO}_3)-1\rightarrow3-\alpha-L-Fucp-4(\text{OSO}_3)-1]_n\). The egg jellies of these two species of sea urchins induce the acrosome reaction in homologous but not in heterologous sperm. Therefore, the fine structure of the sulfated \(\alpha\)-fucans from the egg jelly of *S. pallidus* and *S. droebachiensis*, which differ in their sulfation patterns and in the position of their glycosidic linkages, ensures species-specificity of sperm acrosome reaction and prevents interspecific crosses. In addition, our observations allow a clear appreciation of the common structural features among the sulfated polysaccharides from the egg jelly of sea urchins and help to identify structures that confer finer species-specificity of recognition for the acrosome reaction.
INTRODUCTION

Broadcast spawning echinoderms are a model system for studying molecular mechanisms of fertilization and the evolution of mating barriers. In marine species without temporal or spatial segregation of spawning events, molecular recognition of egg and sperm surfaces is critical to prevent hybridization. Knowing which steps confer species-specificity will further our understanding of the evolution of reproductive isolation and ultimately of speciation and biodiversity. Environmental spawning cues and sperm attractants have not been found to be species-specific in sea urchins (1). Species-specificity must therefore be achieved during subsequent gamete interactions. Once released, the sperm must find and interact with an egg of the correct species. An obligatory event for sperm binding to, and fusion with, the egg is the induction of the acrosome reaction in the sperm, an exocytosis of lytic and binding proteins from a vesicle at the tip of the sperm head. This is a signal transduction event linked to ion fluxes, membrane depolarization and internal pH changes, but whose signal transduction pathway remains to be elucidated (2,3).

The sea urchin egg is surrounded by a transparent jelly coat, which contains molecules inducing physiological changes in sperm (4). A major macromolecule of the egg jelly coat, the one responsible for inducing the sperm acrosome reaction, is a sulfated polysaccharide (5-7). We have demonstrated that these compounds have simple, repeating structures and each species represents a particular pattern of sulfate substitution. The sulfated polysaccharides are species-specific as inducers of the sperm acrosome reaction (5) and represent an unusual simple example of ligand-induced signal transduction leading to exocytosis (5,8).

We also reported two structurally distinct sulfated α-L-fucans in the egg jelly of the sea urchin Strongylocentrotus purpuratus (6). Approximately 90% of individual females of
this species spawn eggs having only one of two possible fucans. Both purified sulfated \(\alpha\)-L-fucans have equal potency in inducing acrosome reaction in homologous sperm. The reason that eggs from this species possess two sulfated fucan isotypes remains unknown.

For our initial demonstration that sulfated polysaccharides are species-specific inducers of the acrosome reaction, we used polysaccharides from distantly related species expressing marked interspecific structural variation (5). More recently we evaluated the finer specificity of recognition in the acrosome reaction with egg jelly sulfated fucans containing the same backbone of 3-linked \(\alpha\)-L-fucopyranosyl units, but with different proportions of 2-\(O\)- and 4-\(O\)-sulfation (7). Although we observed a less strict species-specificity in sperm recognition of sulfated polysaccharides, the potency of acrosome reaction induction clearly depends on the extent of 2-\(O\)- and 4-\(O\)-sulfation in the chain of 3-linked \(\alpha\)-L-fucopyranosyl units (7).

Here we extend our studies to two new sea urchins, the closely related species *Strongylocentrotus droebachiensis* and *S. pallidus*, which both have a circumarctic distribution. The egg jellies of these sea urchins contain sulfated \(\alpha\)-fucans with new structures. Our results show expanded possibilities for structural variation among sulfated \(\alpha\)-L-fucans from echinoderms and possible biological and evolutionary implications of these unique polysaccharides. Detailed structural characterizations also help evaluate the therapeutic potential of sulfated polysaccharides, as already demonstrated for the anticoagulant activity of sulfated fucans (9) and sulfated galactans (10).
EXPERIMENTAL PROCEDURES

Sulfated fucans from the sea urchin egg jelly

*Extraction*-Mature female of *Strongylocentrotus droebachiensis* and *S. pallidus* were collected near Friday Harbor, WA, USA. Atlantic *S. droebachiensis* were collected in Bergen, Tromsø, and Svalbard in Norway. Eggs were spawned into filtered sea water after intracelomic injection of 0.55 M KCl. Egg jelly was isolated by pouring eggs repeatedly through nylon mesh and prepared as 20,000 x *g* supernatant and stored at -20°C, or lyophilized after dialysis against distilled water (8). The acidic polysaccharides were extracted from the jelly coat by papain digestion and partially purified by ethanol precipitation, as described previously (11).

*Purification*-The crude polysaccharides (10 mg) from the egg jelly coats were applied to a Mono Q column-FPLC (HR 5/5) (Pharmacia Biotech Inc.) equilibrated in 20 mM Tris:HCl (pH 8.0). The column was washed with 10 mL of the same buffer and then eluted by a linear gradient of 0 - 4.0 M NaCl in the same buffer. The flow rate of the column was 0.45 mL/min, and fractions of 0.5 mL were collected. Fractions were checked for fucose and sialic acid by the Dubois *et al.* reaction (12) and by the Ehrlich assay (13), respectively, and by their metachromasia (14). The NaCl concentration was estimated by conductivity. Fractions containing the sulfated α-L-fucan and the sialic acid-glycoconjugate were pooled, dialyzed against distilled water, and lyophilized.

*Chemical analyses*-Total fucose was measured by the method of Dische and Shettles (15). After acid hydrolysis of the polysaccharide (5.0 M trifluoroacetic acid for 5 h at 100°C), sulfate was measured by the BaCl₂/gelatin method (16). The presence of hexoses and 6-deoxyhexoses in the acid hydrolysates were estimated by paper

Agarose gel electrophoresis—Sulfated fucans were analyzed by agarose gel electrophoresis as described previously (5,18). The sample (~15 µg) was applied to a 0.5% agarose gel and run for 1 h at 110 V in 0.05 M 1,3-diaminopropane:acetate (pH 9.0). The sulfated polysaccharides in the gel were fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide solution. After 12 h, the gel was dried and stained with 0.1% toluidine blue in acetic acid:ethanol:water (0.1:5:5, v/v).

Desulfation and methylation of the fucans—Desulfation of the sulfated fucans was performed by solvolysis in dimethylsulfoxide as described previously for desulfation of other types of polysaccharides (19,20). Sulfate esters located at different sites of the fucose residues may have variable susceptibility to the desulfation reaction (5-7). In addition, desulfation reaction simultaneously reduced the molecular mass of the polysaccharide. It is necessary to have a balance between removal of sulfate ester and decrease of the polysaccharide chain. For these reasons we obtained in some experiments a total desulfated and in others a partially desulfated fucan.

The native and desulfated fucans (5 mg of each) were subjected to three rounds of methylation as described previously (21), with the modifications suggested by Patankar et al. (22). The methylated polysaccharides were hydrolyzed in 6 M trifluoroacetic acid for 5 h at 100°C, reduced with borohydride and the alditols were acetylated with acetic anhydride:pyridine (1:1, v/v) (17). The alditols acetates of the methylated sugars were dissolved in chloroform and analyzed in a gas chromatography/mass spectrometer.
NMR experiments—1H and 13C spectra of the native and desulfated fucans were recorded using a Bruker DRX 600 with a triple resonance probe. About 3 mg of each sample was dissolved in 0.5 mL of 99.9% D2O (CIL). All spectra were recorded at 60°C with HOD suppression by presaturation. COSY, TOCSY and 1H/13C heteronuclear correlation (HMQC) spectra were recorded using states-TPPI (states-time proportion phase incrementation) for quadrature detection in the indirect dimension. TOCSY spectra were run with 4,096 x 400 points with a spin-lock field of about 10 KHz and a mixing time of 80 ms. HMQC were run with 1,024 x 256 points and GARP (globally optimized alternating phase rectangular pulses) for decoupling. NOESY spectra were run with a mixing time of 100 ms. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for 1H and to methanol for 13C.

Fertilization—Sea urchins were induced to spawn by intracoelomic injection of 0.55 M KCl. Sperm were collected undiluted from the gonopores and stored on ice, while eggs were released into filtered seawater at ambient water temperature. Freshly diluted sperm were added to 480 μL aliquots of gently washed 5% egg suspensions (v/v) in 24-well tissue culture plates. A 1:4 dilution of sperm at each of five steps, starting with a 1:10,000 dilution of sperm, covered the range from near zero to 100% fertilization for intraspecific crosses. Fertilization success was assessed by counting the proportion of eggs, out of 200-300 per well, with an elevated fertilization envelope or of eggs that were cleaving. The concentration of sperm, which differs among species and individuals, was determined later by ten counts of fixed sperm suspensions in a hemocytometer. The percentages of fertilization were calculated by backtransformation from logistic regressions for multiple male/female combinations crossed over a range of sperm concentrations.

To obtain egg jelly for acrosome-reacting sperm, a 5-10% suspension of eggs was poured through nitex mesh several times. This stripped the eggs of their soluble jelly; the
supernatant was pipetted off after the dejellied eggs had settled. Carrying out the final sperm dilution step in conspecific egg jelly water induced the sperm acrosome reaction and is referred to as “pre-reaction with conspecific egg jelly”.
RESULTS AND DISCUSSION

Egg jellies of the sea urchin *S. droebachiensis* but not those of *S. pallidus* possess two isotypes of sulfated fucans. Agarose gel electrophoresis in 1,3 diaminopropane:acetate buffer followed by toluidine blue staining showed that egg jelly, isolated from individual females of *East Pacific S. droebachiensis* contained either a slow (sulfated fucan I) or fast (sulfated fucan II) migrating fucan isotype (Fig. 1A). Of 22 individual females, 9 had eggs with sulfated fucan I and 13 had eggs with sulfated fucan II. Surprisingly, 9 individual females of the same species but collected in the Atlantic Ocean contained only the slow migrating sulfated fucan (isotype I) (Fig. 1B).

Small differences in the electrophoretic mobility of sulfated fucans I and II (Fig. 1A,B) could reflect intermediate sulfation degrees, variation in the molecular mass of the polymers (11,23) or even interaction of the sulfated fucan with other macromolecules (24) since the agarose gel electrophoresis was performed with crude egg jelly. These aspects were further investigated using Mono Q-FPLC chromatography of mixed samples of egg jellies from a large number of *S. droebachiensis* females. Egg jellies from 31 Pacific females showed two distinct fractions of sulfated fucans (Fig. 2A) while egg jellies from 19 Atlantic females contained a single fraction eluted at lower NaCl concentration (Fig. 2B). A peak rich in sialic acid was eluted completely by 0.7 M NaCl from the two samples and denominated as “sialic acid-rich glycoconjugate” in analogy with similar compounds described in other species of sea urchin (25).

The absence of intermediate fractions between sulfated fucans I and II suggests that females of *S. droebachiensis* synthesize either type of fucan with a defined sulfation pattern. If the difference between the sulfated fucans from females of *S. droebachiensis* was a consequence of temporal variation in the sulfation or related to the stage of oogenesis, one would expect to see intermediate fractions between sulfated fucan I and
sulfated fucan II in the agarose gel electrophoresis (Fig. 1A,B) and on the anion exchange chromatography (Fig. 2A,B).

Seven individual females of the sea urchin *S. pallidus* from the Pacific coast, collected at the same site as the *S. droebachiensis* females used in the experiment of Fig. 1A, contained a single sulfated fucan isotype (Fig. 1C). Mono Q-FPLC chromatography of a mixed sample of egg jellies from 25 *S. pallidus* females confirmed the occurrence of a single sulfated fucan (Fig. 2C) eluted at high NaCl concentration, just as the sulfated fucan II from *S. droebachiensis*, besides the “sialic acid-rich glycoconjugate”.

Overall, these results indicate that spawned eggs from individual females of the sea urchin *S. droebachiensis* have one of two possible sulfated fucan isotypes. This polymorphism was observed only in one population. In contrast, all assayed females of the sea urchin *S. pallidus* contained a single type of sulfated fucan.

*Sulfated α-fucans from S. droebachiensis are linear 4-linked polysaccharides but differ in the extension of their 2-O-sulfation*-Both sulfated fucans, purified as in Fig. 2A,B, migrated on agarose gels (Fig. 3) identically as crude egg jelly shown in Fig. 1A,B. The slow and fast migrating sulfated fucans were eluted at low and high NaCl concentrations, respectively. Chemical analysis of the purified sulfated fucans (Table I) revealed fucose as the only sugar with a high content of sulfate ester, which increased from sulfated fucan I to sulfated fucan II, as expected from their migration on the agarose gel electrophoresis (Fig. 1A) and elution from the anion exchange chromatography (Fig. 2A).

Methylation of the native sulfated fucan I from *S. droebachiensis* yielded equimolar proportions of 2,3-di-<chem>O-methyl</chem>-fucose and 3-methyl-fucose, whereas 2,3-di-<chem>O-methyl</chem> fucose was the predominant methyl ether derivative from desulfated fucan I (Table II). This indicates a polysaccharide composed of 4-linked fucopyranoside residues, partially 2-
This structure was confirmed and further detailed by NMR analysis. The $^1$H 1D and $^1$H/$^1$H HMQC spectra of the native and desulfated fucan I from *S. droebachiensis* are shown in Figs. 4A,B and 5A,B, respectively. The chemical shifts in Table III are based on the interpretations of TOCSY, COSY and HMQC spectra.

NMR spectra of the desulfated fucan I showed a single anomic signal (Fig. 4B) with a strong downfield shift (~11 ppm) of C4 (Fig. 5B and Table III), compatible with a linear homopolymer of 4-linked, α-fucopyranoside residues. NMR spectra of native sulfated fucan I contain four anomic signals in near equal proportions by integration (Fig. 4A and 5A). TOCSY and COSY spectra confirmed that the four anomic signals of native sulfated fucan I correspond to four spin systems, each consistent with α-fucose. The spin systems can be traced giving the values of Table III. Strong downshifts (~0.65 ppm) of H2 of residues A and B relative to H2 of C and D indicate that two of the residues are sulfated at C2. Thus, the sulfated α-fucan I from *S. droebachiensis* is mostly a tetrasaccharide repeat unit consisting of 4-linked residues, two sulfated at the O-2 position and two that are unsulfated.

The order of the four residues can be easily deduced. The only possible array is two consecutive 2-O-sulfated residues followed by two unsulfated. If the 2-O-sulfated and unsulfated units alternate, the fucan would contain a disaccharide instead of a tetrasaccharide repeating structure. Our proposition was confirmed by the NOESY spectrum (Fig. 6). As in the NOESY spectra of other fucans from echinoderms (5,26,27), NOEs between protons of different units can be seen and they were used to reveal the sequence (besides, of course, NOEs to other protons in the same residue). In the sulfated

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1 An additional round of methylation did not increase the proportion of 2,3-di-O-methyl fucose. Possibly the sample still contains small amounts of 2-O-sulfate ester. A different sample of desulfated fucan I was used for NMR analysis.
α-fucan I from *S. droebachiensis*, H1 of residue A showed cross-peaks to H4 of residue B; H1 of B shows cross-peaks to H4 of residue C; H1 of C to H3 of D and H1 of D to H2 of A. This evidence indicates the sequence and linkage $[-4\text{-}A\text{-}1\rightarrow4\text{-}B\text{-}1\rightarrow4\text{-}C\text{-}1\rightarrow4\text{-}D\text{-}1\rightarrow]$, as shown in Fig. 7A.

The presence of minor random component in the sulfated α-fucan I cannot be ruled out. For example, small amounts of three consecutive 2-0-sulfated fucose units followed by three unsulfated residues may occur in the polysaccharide. In this case, the additional structures are either undetectable due to their low proportions or cannot be discriminated by the NMR spectra. Nevertheless, the near equal proportions by integration of the four anomeric signals (Fig. 4A and 5A) indicate these additional structures cannot account for substantial proportion of the sulfated fucan structure.

The structure of sulfated α-fucan II from *S. droebachiensis* was investigated using the same methodologies. Methylation of the native sulfated fucan II yielded 3-methylfucose, whereas 2,3-di-0-methylfucose is obtained from the totally desulfated fucan II (Table II). Clearly this indicates a linear homopolymer composed of 4-linked and 2-0-sulfated fucopyranoside residues, whose structure was confirmed by NMR analysis (Fig. 4C,D and Fig. 5C,D). The $^1$H spectrum of the sulfated α-fucan II resulting from desulfation processes showed a reduction in intensity of the anomeric residue at 5.30 ppm and a corresponding increase at 5.05 ppm.\(^2\) Again, the chemical shifts were based on the interpretations of TOCSY, COSY (not shown) and HMQC spectra (Fig. 5C,D). The chemical shifts of the desulfated residues from fucans I and II are similar, indicating both polysaccharides have the same saccharide backbone. But, in contrast with sulfated fucan

\(^2\) Different samples of desulfated fucan II were used for the methylation and NMR analysis. A totally desulfated fucan II was employed for the methylation analysis (Table II) while a partially desulfated preparation was used for NMR analysis (Fig. 4D and 5D).
I, sulfated fucan II is totally 2-O-sulfated. It contains a single spin system and alterations on desulfation are consistent with 2-O-sulfation: -0.65 ppm for H2, -0.14 for H3 and -0.07 for H3; -6.6 ppm for C2 and +1.1 for C3 (Fig. 4C,D, Fig. 5C,D and Table III).

In conclusion, methylation and NMR analyses indicate that sulfated fucan I and sulfated fucan II from *S. droebachiensis* are linear polysaccharides composed of $\alpha(1\rightarrow4)$ fucopyranose. The two fucans differ in their sulfation pattern. Sulfated fucan I consists mostly of a regular sequence of 4 residues, as follows: $[4-\alpha-L-Fucp-2(OSO_3)-1\rightarrow4-\alpha-L-Fucp-2(OSO_3)-1\rightarrow4-\alpha-L-Fucp-1\rightarrow4-\alpha-L-Fucp-1]_n$ whereas sulfated fucan II is a homopolymer of $[4-\alpha-L-Fucp-2(OSO_3)-1]$ units (Fig. 7A,B). In addition, NMR analyses show the absence of intermediate fractions between sulfated fucans I and II and confirm these two polysaccharides have a well-defined repeating unit determined by specific patterns of sulfation.

*The sulfated $\alpha$-fucan from S. pallidus has a 3-linked tetrasaccharide repeating unit defined by specific pattern of sulfation at the 2-O- and 4-O positions.* The sulfated fucan from *S. pallidus* eluted from an anion exchange chromatography with high NaCl concentration (Fig. 2C), contains fucose as the only sugar with a high content of sulfate ester (Table I) but has a slower mobility on agarose gel electrophoresis than the two sulfated fucans from *S. droebachiensis* (Fig. 3). The electrophoretic mobility of sulfated polysaccharides in 1,3-diaminopropane:acetate buffer depends on the structure of the glycan, which forms a complex with the diamino groups (20,28). Thus, the retarded electrophoretic mobility of the sulfated fucan from *S. pallidus* is a preliminary indication of its distinctive polysaccharide structure.

As in the case of the polysaccharides from *S. droebachiensis*, the structure of this new sulfated fucan was determined by NMR analysis. The native sulfated fucan showed
four anomeric residues in near equal proportions by integration (Fig. 8A and Fig. 9A) while after desulfation a single anomeric signal is seen (Fig. 8B and Fig. 9B), as already observed for sulfated fucan I from *S. droebachiensis* (Fig. 4A, B and Fig. 5A, B). But, in the case of desulfated fucan from *S. pallidus*, a strong downfield shift (~8 ppm) of C3 (values in italics, Table IV), and not of C4, is compatible with a 3-linked polysaccharide. The NMR spectra of the native sulfated fucans from the two species of sea urchins also differ significantly. For *S. pallidus* strong downshifts of H2 of residues A and B (-0.50 ppm) and of H4 of residues C and D (-0.70 ppm) (Fig. 10A, Table IV) indicate that two of the four residues are 2-\(O\)-sulfated and the other two are 4-\(O\)-sulfated. Minor structural components, which may occur in this sulfated \(\alpha\)-fucan (such as those indicated by arrows in Fig. 8A) do not account for more than 5% of the total signals in the anomeric region, based on integration of the peaks in this region of the \(^1\)H spectrum. In addition, the proportions of these minor components but not those of the A-D spin systems vary among different preparations of sulfated \(\alpha\)-fucan.

The order of the four residues can be easily deduced, as already discussed for sulfated fucan I from *S. droebachiensis*. The only possible array is two consecutive 2-\(O\)-sulfated residues followed by two 4-\(O\)-sulfated residues. Again, if the 2-\(O\)- and 4-\(O\)-sulfated units alternate, the fucan would contain a disaccharide instead of a tetrasaccharide repeating structure. There is no indication of disulfated units in the TOCSY. Although only one interresidue NOEs could be unambiguously identified in the NOESY spectrum (Fig. 10B) it was enough to confirm the proposed structure. NOEs from H1 of residue A to H4 of residue D while H1 of residues B, C or D do not have any interresidue NOEs. These NOEs are in agreement with the repeating unit of this sulfated fucan as -B-A-D-C- (Fig. 7C).
Overall, the NMR analyses indicate the sulfated fucan from *S. pallidus* is composed mostly of a regular sequence of four residues, as follows: [3-α-L-Fucp-2(OSO₃)₂-1→3-α-L-Fucp-2(OSO₃)₂-1→3-α-L-Fucp-4(OSO₃)₂-1→3-α-L-Fucp-4(OSO₃)₂-1]ₙ (Fig. 7C). As in the case of the sulfated α-fucan I from *S. droebachiensis*, we can not rule out the occurrence of minor random component in the sulfated α-fucan from *S. pallidus*. In this case the additional structures are either undetectable due to their low proportions or cannot be discriminated by the NMR spectra.

**Summary on variants of sulfated α-L-fucans from the egg jelly of sea urchins**—A variety of sulfated fucans were described in marine algae (29-31). These compounds are among the most abundant and widely studied of all sulfated polysaccharides of non-mammalian origin. The algal fucans have complex, heterogeneous structures. Their regular repeating sequences are not easily deduced; even high-field NMR is at the limit of its resolution, and complete description of their structure is not available at present (9,27). Recently we isolated and characterized several sulfated α-L-fucans from echinoderms, mostly from the egg jelly of sea urchins. In contrast to the algal fucans, these sea urchin polysaccharides have simple, linear structures, composed of well-defined repeating units of oligosaccharides (5-7).

The specific pattern of sulfation and the position of the glycosidic linkage vary among sulfated α-L-fucans from different species of sea urchins. *S. droebachiensis* (sulfated α-L-fucan I) and *Arbacia lixula* (5) have a 4-linked sulfated α-L-fucan with the same tetrasaccharide repeating sequence (Fig. 7A). *S. pallidus* and *Lytechinus variegates* (5,27) have 3-linked sulfated α-L-fucans with tetrasaccharide repeating units which differ by specific patterns of sulfation (Fig. 7C and F, respectively). *S. purpuratus* has two structures, found in different individuals: a monosaccharide with variable sulfation at one
position (sulfated α-L-fucan I) and a trisaccharide repeating sequence (sulfated α-L-fucan II) (Fig. 7D and E, respectively) (6). *S. droebachiensis* (sulfated fucan II), *Strongylocentrotus franciscanus* (7), and *Echinometra lucunter* (5) (Fig. 7B, G and H, respectively) have polysaccharides with a single 2-O-sulfated monosaccharide unit, which differ either in the position of their glycosidic linkage or in their constituent monosaccharide. *S. droebachiensis* (sulfated fucan II) and *S. franciscanus* contain 4-linked and 3-linked α-L-fucopyranose, respectively, while *E. lucunter* has 3-linked α-L-galactopyranose.

*Structural features in the sea urchin polysaccharides that confer finer specificity of recognition in the sperm acrosome reaction*-Sulfated polysaccharides from the egg jelly of sea urchins are responsible for inducing the sperm acrosome reaction, which is an obligatory event for fertilization (5-7). Shortly after fertilization the sulfated α-fucan disappears (32), which indicates it has no further role in the embryo development. These polysaccharides are species-specific as inducers of the sperm acrosome reaction and may represent one of the barriers which prevent interspecific fertilization.

We have now fully characterized eight sulfated polysaccharides from the egg jelly of seven species of sea urchins (Fig. 7). We can now formulate questions, such as: what are the common structural features among these polysaccharides? Can we identify the structures that confer finer specificity of recognition for the acrosome reaction?

Clearly, as we examine the eight structures shown in Fig. 7, the common feature shared by these polysaccharides is always the occurrence of 2-O-sulfation at the first unit of the oligosaccharide repeating sequence. In this way, the sea urchin *S. franciscanus*, which contains a sulfated fucan composed exclusively of the common 2-O-sulfated α-L-fucose unit (Fig. 7G), has a less strict species-specificity in sperm recognition of sulfated
polysaccharide. The potency of acrosome reaction induction clearly depends on the extent of 2-\(O\)-sulfation in the chain of 3-linked \(\alpha\)-fucose units (7).

As a distinctive feature for a different polysaccharide backbone, the sea urchin \textit{E. lucunter} synthesizes sulfated \(\alpha\)-L-galactan (Fig. 7H) instead of sulfated \(\alpha\)-L-fucan (5). However, the majority of the sea urchin species contain sulfated \(\alpha\)-fucans with increased complexity due to variable 2-\(O\)- and 4-\(O\)-sulfation of their repetitive oligosaccharide units, as well as the glycosidic linkage at 1\(\rightarrow\)3 or 1\(\rightarrow\)4 positions. In the case of a species enriched in 4-\(O\)-sulfated units, as exemplified by \textit{S. purpuratus} (Fig. 7D,E), a more strict species-specificity was observed than in \textit{S franciscanus}, and the sperm react only with homologous polysaccharide or, to a lesser extent, with heterologous 3-linked fucans enriched in 4-\(O\)-sulfated residues (7).

The two new species of sea urchins we now studied allow a more in depth analysis concerning the species-specificity of sulfated \(\alpha\)-fucans as inducers of the acrosome reaction in echinoderms. The sulfated \(\alpha\)-fucans from these species contain two consecutive 2-\(O\)-sulfated fucose residues, which alternate either with two non-sulfated or 2-\(O\)-sulfated residues (in \textit{S. droebachiensis}) or with two 4-\(O\)-sulfated fucose units (in \textit{S. pallidus}). Therefore, analysis of the species-specificity of the acrosome reaction between these two species will definitively demonstrate that the arrangement of the oligosaccharide repetitive unit determines the sperm reactivity.

In our previous studies we quantified the proportion of sperm that have undergone the acrosome reaction after incubation with sulfated polysaccharides using microscopic examination (5-7). This approach is not possible in the case of the new species of sea urchins due to the extremely pointed tip of \textit{S. droebachiensis} sperm. We overcame this limitation by measuring fertilization successes among three species of \textit{Strongylocentrotus} (Table V). We were able to identify the contribution of sperm acrosome reaction for the
interspecific fertilization of these species by comparison between the ratio of fertilization success after and before pre-reaction of the sperm with conspecific egg jelly. For conspecific fertilization this ratio is ~1.0, as expected, but increases up to 3.67 and 6.67 in the heterospecific crosses. This indicates the induction of sperm acrosome reaction by the egg jelly sulfated fucan is the major limitation for interspecific fertilization between *S. droebachiensis* and *S. pallidus*. Sperm of *S. pallidus* are slightly more potent than those of *S. droebachiensis* to achieve heterospecific fertilization, without pre-activated sperm, and indicate a slightly lower species-specificity (Table V). We cannot distinguish if this is a consequence of differences in the position of the glycosidic linkage (3-linked in *S. pallidus* and 4-linked in *S. droebachiensis*) or in the sulfation pattern of the repetitive tetrasaccharide unit.

For eggs of *S. purpuratus* we still did not detect fertilization after pre-reaction of the sperm with conspecific egg jelly. Therefore, additional steps of gamete interaction, besides induction of the sperm acrosome reaction, prevent interspecific fertilization of *S. purpuratus* eggs by *S. droebachiensis* or *S. pallidus* sperm. For example, the binding of sperm to the eggs could be prevented by divergent evolution of the protein bindin (see Ref. 33 and other references therein).

Overall, the experiments summarized in Table V indicate that the sulfated α-fucans from the egg jelly of *S. pallidus* and *S. droebachiensis* induce the acrosome reaction in homologous but not in heterologous sperm. This was confirmed by recent assays of acrosomal exocytosis using immunofluorescence microscopy and bindin antibody.\(^3\) Again, the immunological staining of sperm after incubation with the purified sulfated α-fucans demonstrated that the egg jelly polysaccharides induce acrosome reaction in homologous but not in heterologous sperm. This is the major limitation for interspecific fertilization

\(^3\) Biermann, C.H., unpublished results.
between these two species of sea urchins. It is interesting, and suggestive of adaptation, that these two closely related species, which co-occur over a huge geographic range, show such a strong specificity early on in the cascade of gamete recognition events.

Two sulfated α-fucan isotypes in a single species of sea urchin—We now extended to *S. droebachiensis* our observation in *S. purpuratus* (6) that individual females spawn eggs possessing only one of two sulfated α-L-fucan isotypes (Fig. 1 A,B). As in *S. purpuratus*, both *S. droebachiensis* isotypes induce the acrosome reaction with similar potency in homologous sperm, as revealed by the immunofluorescence microscopy assay. It appears that in *S. droebachiensis*, one of the isotypes does not occur, or occurs at lower frequencies, in a population from a different ocean. Additional studies, with a larger number of females and collected at a variety of geographic sites, are necessary to further clarify the role of genetic or environmental factors.

The two sulfated α-fucans isoforms of *S. droebachiensis* have well-defined sulfation patterns and are not a consequence of variable degree of sulfation (Fig. 7A,B). The inheritance of such sulfation patterns is unknown. We expected that they are produced by site specific sulfotransferases, by analogy with the extensive studies on the biosynthesis of mammalian glycosaminoglycans. Sulfated fucan II requires a single sulfotransferase. But, sulfated fucan I requires two sulfotransferases, one that recognizes the first α-fucose residue of the repeating sequence and a second that recognizes the 2-O-sulfated fucose unit and sulfates the second residue.\(^4\) Of course we cannot exclude

\(^4\) In the case of *S. pallidus* two additional sulfotransferases may be involved in the biosynthesis of the sulfate fucan. One to recognize the two consecutive 2-O-sulfated fucose units and then sulfate C4 of the third residue, and another transferase to recognize
unique metabolic pathways, as reported for the biosynthesis of a sulfated α-L-galactan from ascidian (34,35). For example, an alternative to explain the presence of either sulfated fucan I or sulfated fucan II in separate females of *S. droebachiensis* is to postulate that in both types of females all fucose residues become 2-O-sulfated, but in the females containing sulfated fucan I, specific sulfatases remove the sulfate esters from the third and fourth residues.

Another noteworthy observation is that *S. droebachiensis* and *A. lixula*, unrelated sea urchin species from the Arctic and tropical Atlantic Oceans, respectively, synthesize sulfated α-fucans with the same repetitive structure (Fig. 7A). Recent experiments with immunological staining of *S. droebachiensis* sperm with bindin antibody after incubation with the purified polysaccharides indicate that *A. lixula* sulfated fucan is indeed equivalent to *S. droebachiensis* sulfated fucan I in its physiological activity *in vitro*. According to phylogenetic analysis these two species diverged ~200 million years ago (36). The species *S. droebachiensis*, *S. pallidus* and *S. purpuratus* diverged 3.5 million years ago (37) but their egg jelly sulfated fucans are markedly different. Therefore, the genes involved in the biosynthesis of the sulfated fucans and their sperm receptors (8) did not evolve in concordance with the evolutionary distance between these echinoderms but were possibly driven to diverge by natural selection where several species co-occur.

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the sulfation pattern of the first three fucose residues and then to sulfate the fourth unit at the C4 position to obtain the repeating sequence shown in Fig. 7C.
Acknowledgments—The authors are grateful to Adriana A. Piquet for technical assistance, to an anonymous reviewer, and to Friday Harbor Laboratories for supporting Christiane Biermann. Very special thanks to Jessica Marks for Norwegian sea urchins. This work is part of a DSc thesis submitted to the Departamento de Bioquímica Médica, Universidade Federal do Rio de Janeiro by Ana-Cristina E.S. Vilela-Silva.
REFERENCES


Table I

*Chemical composition of the sulfated α-fucans from the egg jelly of two sea urchin species in the genus Strongylocentrotus*

<table>
<thead>
<tr>
<th>Species</th>
<th>Polysaccharide</th>
<th>Fucose</th>
<th>Sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. droebachiensis</em></td>
<td>Sulfated fucan I</td>
<td>1.00</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Sulfated fucan II</td>
<td>1.00</td>
<td>0.80</td>
</tr>
<tr>
<td><em>S. pallidus</em></td>
<td></td>
<td>1.00</td>
<td>1.30</td>
</tr>
</tbody>
</table>

*Molar ratios*

*aPurified sulfated α-fucans I and II from *S. droebachiensis* were obtained by Mono Q-FPLC chromatography (see Fig. 2A,B). Sulfated α-fucan from *S. pallidus* was purified as shown in Fig. 2C.*
Table II

*Methylated derivatives obtained from native and desulfated fucans from the egg jelly of Strongylocentrotus droebachiensis*

<table>
<thead>
<tr>
<th>Alditols&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$t_R$&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sulfated fucan I</th>
<th>Native</th>
<th>Desulfated</th>
<th>Sulfated fucan II</th>
<th>Native</th>
<th>Desulfated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-Me&lt;sub&gt;2&lt;/sub&gt;-Fuc</td>
<td>25.8</td>
<td>49</td>
<td>71</td>
<td>&lt;1</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Me-Fuc</td>
<td>30.1</td>
<td>51</td>
<td>29</td>
<td>100</td>
<td>&lt;1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The identity of each peak was established by mass spectrometry.

<sup>b</sup>Retention time on a DB-1 capillary column.
Table III

Proton and carbon chemical shifts (ppm) for residues of α-fucose in native and chemical desulfated fucans from *S. droebachiensis* *a*

<table>
<thead>
<tr>
<th>Sulfated fucan I</th>
<th>Sulfated fucan II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Native</strong>b</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proton</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Desulfated</th>
<th>Native</th>
<th>Desulfated</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>5.343</td>
<td>5.293</td>
<td>5.094</td>
<td>5.050</td>
<td>5.070</td>
<td>5.300</td>
<td>5.053</td>
</tr>
<tr>
<td>H2</td>
<td><strong>4.596</strong></td>
<td><strong>4.596</strong></td>
<td>3.950</td>
<td>3.887</td>
<td>3.950</td>
<td><strong>4.570</strong></td>
<td>3.917</td>
</tr>
<tr>
<td>H4</td>
<td>4.054</td>
<td>4.054</td>
<td>3.983</td>
<td>3.972</td>
<td>3.970</td>
<td>4.030</td>
<td>3.962</td>
</tr>
<tr>
<td>H6</td>
<td>1.330-1.430</td>
<td>1.330-1.430</td>
<td>1.330-1.430</td>
<td>1.330-1.430</td>
<td>1.370</td>
<td>1.360</td>
<td>1.356</td>
</tr>
</tbody>
</table>

**Carbon**

<table>
<thead>
<tr>
<th></th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>101.97</td>
<td><strong>77.90</strong></td>
<td>70.97</td>
<td><strong>83.40</strong></td>
<td>69.96</td>
<td>13.00</td>
</tr>
<tr>
<td>H2</td>
<td>101.60</td>
<td><strong>77.90</strong></td>
<td>71.30</td>
<td><strong>83.40</strong></td>
<td>69.96</td>
<td>13.00</td>
</tr>
<tr>
<td>H3</td>
<td>103.40</td>
<td>103.03</td>
<td>71.30</td>
<td><strong>83.90</strong></td>
<td>69.96</td>
<td>13.00</td>
</tr>
<tr>
<td>H4</td>
<td>103.03</td>
<td>103.50</td>
<td>71.30</td>
<td><strong>83.90</strong></td>
<td>69.96</td>
<td>13.00</td>
</tr>
<tr>
<td>H5</td>
<td>103.50</td>
<td>103.50</td>
<td>71.30</td>
<td><strong>82.75</strong></td>
<td>70.02</td>
<td>13.00</td>
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<tr>
<td>H6</td>
<td>98.20</td>
<td>74.50</td>
<td>67.94</td>
<td>80.10</td>
<td>66.98</td>
<td>14.00</td>
</tr>
<tr>
<td></td>
<td>99.80</td>
<td>67.94</td>
<td>67.94</td>
<td>79.80</td>
<td>66.78</td>
<td>14.00</td>
</tr>
</tbody>
</table>

* aThe spectra were recorded at 600 MHz in 99.9% D$_2$O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for $^1$H and to methanol for $^{13}$C. Values in boldface indicate positions bearing a sulfate ester and those in italics indicate glycosylated positions.

* bSulfated fucan I contains four types of α-fucose residues (see Fig. 4A and 5A).
Table IV

Proton and carbon chemical shifts (ppm) for residues of α-fucose in native and chemical desulfated fucan from *S. pallidus*<sup>a</sup>

<table>
<thead>
<tr>
<th>Proton</th>
<th>Native&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Desulfated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>H1</td>
<td>5.522</td>
<td>5.430</td>
</tr>
<tr>
<td>H2</td>
<td>4.594</td>
<td><strong>4.602</strong></td>
</tr>
<tr>
<td>H3</td>
<td>4.180</td>
<td>4.180</td>
</tr>
<tr>
<td>H4</td>
<td>4.120</td>
<td>4.120</td>
</tr>
<tr>
<td>H6</td>
<td>1.322-1.430</td>
<td>1.322-1.430</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbon</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>94.50</td>
<td>97.00</td>
<td>97.20</td>
<td>95.70</td>
<td>94.52</td>
</tr>
<tr>
<td>C2</td>
<td><strong>75.90</strong></td>
<td><strong>75.90</strong></td>
<td>70.05</td>
<td>70.05</td>
<td>65.35</td>
</tr>
<tr>
<td>C3</td>
<td>78.55</td>
<td>78.55</td>
<td>78.55</td>
<td>78.55</td>
<td>74.01</td>
</tr>
<tr>
<td>C4</td>
<td>71.80</td>
<td>71.80</td>
<td><strong>81.80</strong></td>
<td><strong>81.80</strong></td>
<td>67.59</td>
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<tr>
<td>C5</td>
<td>69.13</td>
<td>69.13</td>
<td>69.13</td>
<td>69.13</td>
<td>65.23</td>
</tr>
<tr>
<td>C6</td>
<td>15.00</td>
<td>15.00</td>
<td>15.00</td>
<td>15.00</td>
<td>13.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>The spectra were recorded at 600 MHz in 99.9% D<sub>2</sub>O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for <sup>1</sup>H and to methanol for <sup>13</sup>C. Values in boldface indicate positions bearing a sulfate ester and those in italics indicate glycosylated positions.

<sup>b</sup>The sulfated fucan from *S. pallidus* contains four types of α-fucose residues (see Fig. 8A and 9A).
Table V

*Fertilization success of plain sperm and sperm prereacted with egg jelly, for crosses among three* Strongylocentrotus *species.*

<table>
<thead>
<tr>
<th>Sperm from</th>
<th>Eggs from</th>
<th>Pre-reaction with conspecific egg jelly</th>
<th>Mean percentage of eggs fertilized&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ratio of fertilization after and before pre-reaction with conspecific egg jelly</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. droebachiensis</td>
<td>S. droebachiensis</td>
<td>-</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>85</td>
<td>1.06</td>
</tr>
<tr>
<td>S. pallidus</td>
<td></td>
<td>-</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>60</td>
<td>6.67</td>
</tr>
<tr>
<td>S. purpuratus</td>
<td></td>
<td>-</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>S. pallidus</td>
<td>S. droebachiensis</td>
<td>-</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>88</td>
<td>3.67</td>
</tr>
<tr>
<td>S. pallidus</td>
<td></td>
<td>-</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>78</td>
<td>1.07</td>
</tr>
<tr>
<td>S. purpuratus</td>
<td></td>
<td>-</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The values are percent of eggs fertilized at a sperm concentration of 200 sperm/µl, before (-) and after (+) pre-reaction with conspecific egg jelly.
FIGURE LEGENDS

Fig. 1. Agarose gel electrophoresis of the sulfated α-fucans extracted from the egg jelly of different individual females of *S. droebachiensis* or *S. pallidus* from the Pacific or Atlantic Oceans. Sulfated fucans were extracted from the egg jelly of different females using papain digestion and partially purified by ethanol precipitation. The sulfated fucans (~15 µg) were then applied to a 0.5% agarose gel, and the electrophoresis was run for 1 h at 110 V in 0.05 1,3-diaminopropane:acetate (pH 9.0). Gels were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution. After 12 h, the gels were dried and stained with 0.1% toluidine blue in acetic acid:ethanol:water (0.1:1:5,v/v).

Fig. 2. Purification of the sulfated α-fucans from the egg jelly of sea urchins by Mono Q-FPCL chromatography. A mixed sample of sulfated α-fucans from 31 Pacific (*A*) and 19 Atlantic (*B*) *S. droebachiensis* females or from 25 Pacific *S. pallidus* females (*C*) was applied to a Mono Q-FPLC column (HR 5/5), equilibrated with 20 mM Tris/HCl buffer (pH 8.0). The column was developed by a linear gradient of 0 - 4.0 M NaCl in the same buffer. Fractions were assayed by metachromasia using 1,9-dimethylmethylene blue (*●*), Dubois reaction for fucose (*○*) and Ehrlich assay for sialic acid (*▲*). The NaCl concentration was estimated by conductivity (----). Fractions containing the sulfated fucans were pooled, dialyzed against distilled water, and lyophilized. SG indicates “sialic acid-rich glycoconjugate”.

Fig. 3. Agarose gel electrophoresis of the purified sulfated α-fucans from *S. droebachiensis* and *S. pallidus* from the Atlantic or Pacific Oceans. A mixed sample and purified sulfated fucans I and II from *S. droebachiensis* as well as the purified fucan
from *S. pallidus* (15 µg of each) were applied to a 0.5% agarose gel and the electrophoresis was run and stained as described in the legend of Fig. 1. *M*, mixture of sulfated fucans I and II; *SF*, sulfated fucan.

Fig. 4. $^1$H 1D NMR spectra at 600 MHz of the native (*A* and *C*) and desulfated (*B* and *D*) α-fucan I (*A* and *B*) and α-fucan II (*C* and *D*) from *S. droebachiensis*. The spectra were recorded at 60°C for samples in D$_2$O solution. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. The residual water has been suppressed by presaturation. The α-anomers assigned by the $^1$H/$^13$C HMQC (see Fig. 5A) are labeled as *A*, *B*, *C*, and *D* in the native sulfated α-fucan I. Expansion of the 4.9 - 5.5 ppm region of the $^1$H spectrum is shown in the inset in Panel *A*. The integrals listed under the anomeric signals are normalized to a total number of anomeric protons.

Fig. 5. $^1$H/$^13$C HMQC spectra of native (*A* and *C*) and desulfated (*B* and *D*) α-fucan I (*A* and *B*) and α-fucan II (*C* and *D*) from *S. droebachiensis*. The assignment was based on TOCSY and COSY spectra. The values of chemical shifts in Table III are relative to external trimethylsilylpropionic acid at 0 ppm for $^1$H and to methanol to $^13$C. The anomeric signals were identified by the characteristic carbon chemical shifts and are marked as *A*, *B*, *C*, and *D* for native sulfated α-fucan I. The integrals of the anomeric signals A, B and C + D are 0.18, 0.23 and 0.59, respectively.

Fig. 6. Expansion from the NOESY spectrum of the sulfated α-fucan I from *S. droebachiensis*. The four fucose residues in the repeating unit are marked *A*, *B*, *C* and *D* as in Fig. 4A. NOEs from H1 of each residue to the following ring proton, in particular the sequence-defining NOEs A1-B4, B1-C4, C1-D3 and D1-A2.
Fig. 7. **Structures of sulfated α-L-fucans and sulfated α-L-galactan from the egg jelly of sea urchins.** The figure shows 8 fully characterized structures of sulfated polysaccharides from the egg jelly of 7 species of sea urchins. The specific pattern of sulfation, the position of the glycosidic linkage and the constituent monosaccharide vary among sulfated polysaccharides from different species (5-7, 27). See text for details.

Fig. 8. **1H D NMR spectra at 600 MHz of the native (A) and desulfated (B) α-fucan from S. pallidus.** Polysaccharide samples and conditions for NMR spectra were as described in the legend of Fig. 4. Expansion of the 5.0 - 5.6 ppm region of the spectrum is shown in the inset of Panel A. The integral listed under the proton of the spectrum are normalized to a total number of anomeric protons. The arrows in Panel A indicate possible contaminants. The 4 fucose anomeric signals are marked as A, B, C, and D for the native sulfated α-fucan.

Fig. 9. **1H/13C HMQC spectra of native (A) and desulfated (B) α-fucan from S. pallidus.** The assignment was based on TOCSY and COSY spectra and the values of chemical shifts are in Table IV. See additional information about the spectra in the legend of Fig. 5. The anomeric signals were identified by the characteristic chemical shifts and are marked as A, B, C, and D for native sulfated α-fucan. The integrals of the anomeric signals A, B, C and D are 0.25, 0.26, 0.28 and 0.21, respectively.

Fig. 10. **Expansions of the TOCSY (A) and NOESY (B) spectra of the sulfated fucan from S. pallidus.** The TOCSY spectrum (A) shows some cross-peaks used in the assignment of the fucose residue, especially positions bearing sulfate esters. The NOESY
spectrum (B) shows NOEs, especially the sequence-defining A1-D4. The four fucose residues in the repeating unit are marked A, B, C, and D as in Fig. 8.
FIG. 1
Vilela-Silva et al.
Metachromatic property ($A_{525\text{nm}}$, ○), Dubois reaction ($A_{490\text{nm}}$, O) and Ehrlich assay ($A_{525\text{nm}}$, △)

FIG. 2
Vilela-Silva et al.
Sulfated fucan II  
Sulfated fucan I  

Polysaccharide: M SF-I SF-I SF-II SF  
Origin: Atlantic Pacific  
Species: S. droebachiensis S. pallidus

FIG. 3
Vilela-Silva et al.
**Figure 8**

Vilela-Silva *et al.*
FIG. 10
Vilela-Silva et al.
Sulfated fucans from the egg jelly of sea urchins: Sulfated fucans from the egg jelly of the closely related sea urchins Strongylocentrotus droebachiensis and S. pallidus ensure species-specific fertilization

Ana-Cristina E.S. Vilela-Silva, Michelle O. Castro, Ana-Paula Valente, Christiane H. Biermann and Paulo A.S. Mourão

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