

Sulfated Fucans from the Egg Jellies of the Closely Related Sea Urchins *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus* Ensure Species-specific Fertilization*

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Sulfated polysaccharides from 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 examined 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 extent of their 2-O-sulfation. Sulfated fucan I is mostly composed of a regular sequence of four residues ($[4-\alpha\text{-L-Fucp-2}(\text{OSO}_3)\text{-1}\rightarrow 4-\alpha\text{-L-Fucp-2}(\text{OSO}_3)\text{-1}\rightarrow 4-\alpha\text{-L-Fucp-1}\rightarrow 4-\alpha\text{-L-Fucp-1}]_n$), whereas sulfated fucan II is a homopolymer of 4- $\alpha\text{-L-Fucp-2}(\text{OSO}_3)\text{-1}$ units. Females of *S. pallidus* contain a single sulfated fucan with the following repeating structure: $[3-\alpha\text{-L-Fucp-2}(\text{OSO}_3)\text{-1}\rightarrow 3-\alpha\text{-L-Fucp-2}(\text{OSO}_3)\text{-1}\rightarrow 3-\alpha\text{-L-Fucp-4}(\text{OSO}_3)\text{-1}\rightarrow 3-\alpha\text{-L-Fucp-4}(\text{OSO}_3)\text{-1}]_n$. The egg jellies of these two species of sea urchins induce the acrosome reaction in homologous (but not heterologous) sperm. Therefore, the fine structure of the sulfated α -fucans from the egg jellies of *S. pallidus* and *S. droebachiensis*, which differ in their sulfation patterns and in the position of their glycosidic linkages, ensures species specificity of the sperm acrosome reaction and prevents interspecies crosses. In addition, our observations allow a clear appreciation of the common structural features among the sulfated polysaccharides from sea urchin egg jelly and help to identify structures that confer finer species specificity of recognition in the acrosome reaction.

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 the signal transduction pathway of which 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 (5–7) that these compounds have simple, repeating structures and that 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 with only one of two possible fucans. Both purified sulfated α -L-fucans have equal potency in inducing the 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 interspecies 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 α -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

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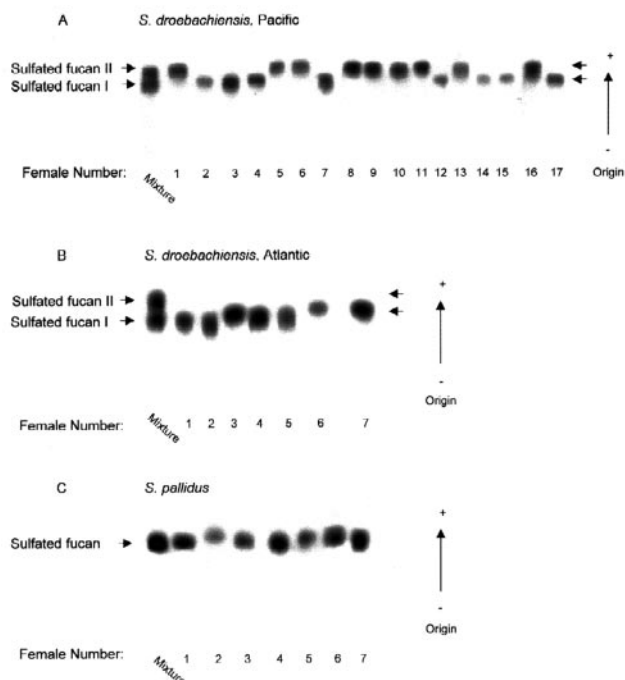


FIG. 1. Agarose gel electrophoresis of the sulfated α -fucans extracted from the egg jellies of different individual females of *S. droebachiensis* and *S. pallidus* from the Pacific and Atlantic Oceans. Sulfated fucans were extracted from the egg jellies of different females using papain digestion and partially purified by ethanol precipitation. The sulfated fucans ($\sim 15 \mu\text{g}$) were then applied to a 0.5% agarose gel, and electrophoresis was carried out for 1 h at 110 V in 0.05 M 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).

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 α -L-fucopyranosyl units (7).

Here, we extend our studies to two new sea urchins, the closely related species *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus*, which both have a circumpolar distribution. The egg jellies of these sea urchins contain sulfated α -fucans with new structures. Our results show expanded possibilities for structural variation among sulfated α -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

Extraction—Mature females of *S. droebachiensis* and *S. pallidus* were collected near Friday Harbor, WA. Atlantic *S. droebachiensis* females were collected in Bergen, Tromsø, and Svalbard, 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, prepared as a 20,000 $\times g$ 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 FPLC¹ column (HR5/5; Amersham Biosciences, Inc.) equilibrated with 20 mM Tris-HCl (pH 8.0). The col-

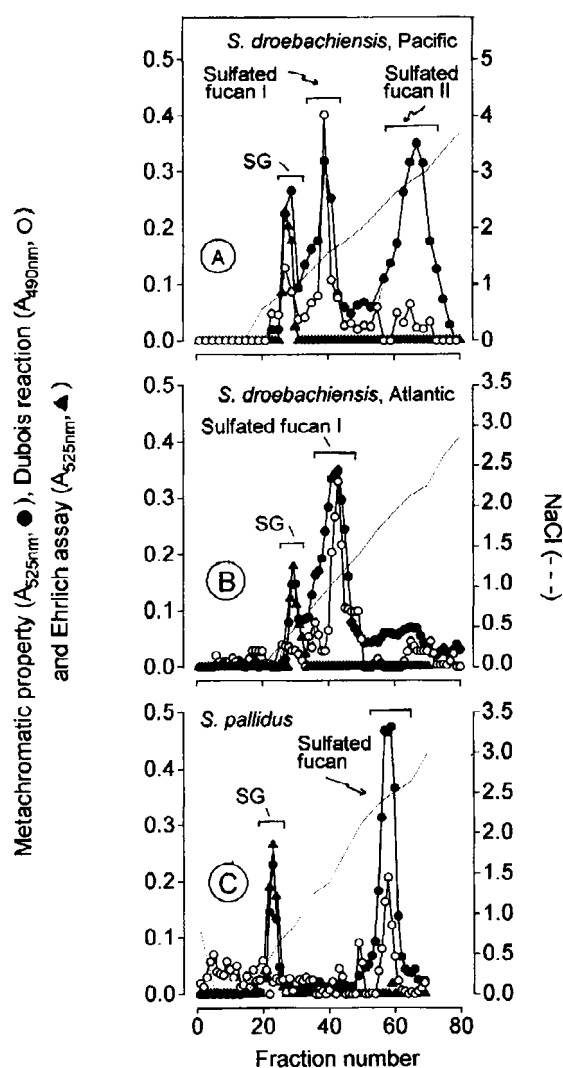


FIG. 2. Purification of the sulfated α -fucans from sea urchin egg jelly by Mono Q FPLC. A mixed sample of sulfated α -fucans from 31 Pacific (A) and 19 Atlantic (B) *S. droebachiensis* females or from 25 Pacific *S. pallidus* females (C) was applied to a Mono Q FPLC column (HR5/5) equilibrated with 20 mM Tris-HCl (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 (●), the Dubois reaction for fucose (○), and the 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.

umn 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 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 BaCl_2 /gelatin method (16). The presence of hexoses and 6-deoxyhexoses in the acid hydrolysates was estimated by paper chromatography in 1-butanol/pyridine/water (3:2:1, v/v) for 48 h and by gas-liquid chromatography-mass spectrometry of derived alditols (17).

Agarose Gel Electrophoresis—Sulfated fucans were analyzed by agarose gel electrophoresis as described previously (5, 18). The sample ($\sim 15 \mu\text{g}$) 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*-trimethylammo-

¹ The abbreviations used are: FPLC, fast protein liquid chromatography; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple quantum correlation spectroscopy; NOESY, nuclear Overhauser effect correlation spectroscopy; NOE, nuclear Overhauser effect.

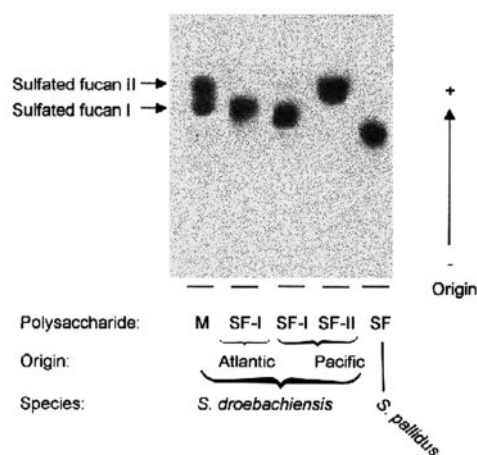


FIG. 3. Agarose gel electrophoresis of the purified sulfated α -fucans from *S. droebachiensis* and *S. pallidus* from the Atlantic and Pacific Oceans. A mixed sample and purified sulfated fucans I and II from *S. droebachiensis* as well as the purified fucan from *S. pallidus* (15 μ g of each) were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

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

Species	Polysaccharide ^a	Chemical composition (molar ratios)	
		Fucose	Sulfate
<i>S. droebachiensis</i>	Sulfated fucan I	1.00	0.53
	Sulfated fucan II	1.00	0.80
<i>S. pallidus</i>		1.00	1.30

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

nium 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 dimethyl sulfoxide 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, the desulfation reaction simultaneously reduced the molecular mass of the polysaccharide. It is necessary to have a balance between removal of sulfate ester and decrease in the polysaccharide chain. For these reasons, we obtained a totally desulfated fucan in some experiments and a partially desulfated fucan in others.

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 and reduced with borohydride, and the alditols were acetylated with acetic anhydride/pyridine (1:1, v/v) (17). The alditol 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 apparatus with a triple resonance probe. About 3 mg of each sample was dissolved in 0.5 ml of 99.9% D₂O Cambridge Isotope Laboratory. All spectra were recorded at 60 °C with HOD suppression by pre-saturation. COSY, TOCSY, and ¹H/¹³C HMQC spectra were recorded using states-time proportion phase incrementation for quadrature detection in the indirect dimension. TOCSY spectra were run with 4096 × 400 points with a spin-lock field of ~10 kHz and a mixing time of 80 ms. HMQC spectra were run with 1024 × 256 points and 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 intracelomic injection of 0.55 M KCl. Sperm were collected undiluted from the

gonophores and stored on ice, whereas eggs were released into filtered seawater at ambient water temperature. Freshly diluted sperm were added to 480- μ l aliquots of gently washed 5% (v/v) egg suspensions 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 eggs/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 10 counts of fixed sperm suspensions in a hemocytometer. The percentages of fertilization were calculated by back-transformation 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 Jelly of the Sea Urchin S. droebachiensis (but Not S. pallidus) Possesses Two Isoforms 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, nine 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. 1, A and 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) because the agarose gel electrophoresis was performed with crude egg jelly. These aspects were further investigated using Mono Q FPLC 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), whereas 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 termed “sialic acid-rich glycoconjugate” in analogy with similar compounds described in other species of sea urchins (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* were a consequence of temporal variation in the sulfation or related to the stage of oogenesis, one would expect to see intermediate fractions between sulfated fucans I and II upon agarose gel electrophoresis (Fig. 1, A and B) and anion-exchange chromatography (Fig. 2, A and 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 in Fig. 1A, contained a single sulfated fucan isotype (Fig. 1C). Mono Q FPLC of a mixed sample of egg jellies from 25 *S. pallidus* females confirmed the occurrence of a single sulfated fucan (Fig. 2C) eluting at high NaCl concentration, like sulfated fucan II from *S. droebachiensis*, in addition to 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 isoforms. This polymorphism was observed only in one population. In contrast, all assayed fe-

TABLE II
Methylated derivatives obtained from native and desulfated fucans from the egg jelly of *S. droebachiensis*

Alditols ^a	t_R^b min	Sulfated fucan I		Sulfated fucan II	
		Native	Desulfated	Native	Desulfated
		% of total peak area		% of total peak area	
2,3-Me ₂ Fuc	25.8	49	71	<1	100
3-MeFuc	30.1	51	29	100	<1

^a The identity of each peak was established by mass spectrometry.

^b Retention time on a DB-1 capillary column.

males 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 Extent of Their 2-O-Sulfation—Both sulfated fucans (purified as in Fig. 2, A and B) migrated on agarose gels (Fig. 3) identically as crude egg jelly (shown in Fig. 1, A and 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 upon agarose gel electrophoresis (Fig. 1A) and elution by anion-exchange chromatography (Fig. 2A).

Methylation of native sulfated fucan I from *S. droebachiensis* yielded equimolar proportions of 2,3-di-*O*-methylfucose and 3-methylfucose, whereas 2,3-di-*O*-methylfucose was the predominant methyl ether derivative from desulfated fucan I (Table II). This indicates a polysaccharide composed of 4-linked fucopyranoside residues, partially 2-*O*-sulfated.² This structure was confirmed and further detailed by NMR analysis. The ¹H one-dimensional and ¹H/¹³C HMQC spectra of native and desulfated fucan I from *S. droebachiensis* are shown in Figs. 4 (A and B) and 5 (A and B), respectively. The chemical shifts in Table III are based on the interpretations of TOCSY, COSY, and HMQC spectra.

NMR spectra of desulfated fucan I show a single anomeric signal (Fig. 4B) with a strong downfield shift (~11 ppm) of C-4 (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 anomeric signals in near-equal proportions by integration (Figs. 4A and 5A). TOCSY and COSY spectra confirmed that the four anomeric 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 in Table III. Strong downshifts (approximately -0.65 ppm) of H-2 of residues A and B relative to H-2 of residues C and D indicate that two of the residues are sulfated at C-2. Thus, 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 residues. 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 on other protons in the same residue). In sulfated α -fucan I

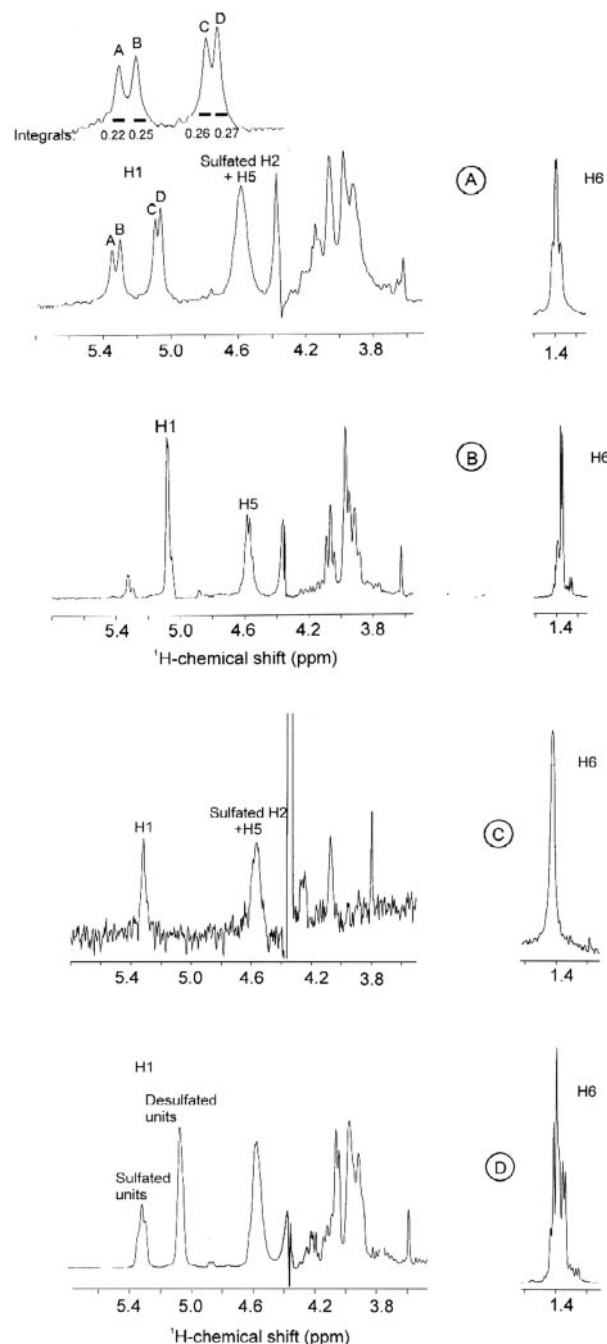


FIG. 4. ¹H one-dimensional NMR spectra at 600 MHz of native (A and C) and desulfated (B and D) α -fucan I (A and B) and α -fucan II (C and D) from *S. droebachiensis*. The spectra were recorded at 60 °C for samples in D₂O solution. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm. The residual water has been suppressed by pre-saturation. The α -anomeric signals assigned by ¹H/¹³C HMQC (see Fig. 5A) are labeled A–D in native sulfated α -fucan I. Expansion of the 4.9–5.5 ppm region of the ¹H spectrum is shown in the inset in A. The integrals listed under the anomeric signals are normalized to a total number of anomeric protons.

² An additional round of methylation did not increase the proportion of 2,3-di-*O*-methyl fucose. Possibly, the sample still contained small amounts of 2-*O*-sulfate ester. A different sample of desulfated fucan I was used for NMR analysis.

TABLE III

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

The spectra were recorded at 600 MHz in 99.9% D₂O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for ¹H and to methanol for ¹³C. Values in boldface type indicate positions bearing a sulfate ester, and those in italic type indicate glycosylated positions.

	Sulfated fucan I				Sulfated fucan II		
	Native ^a				Desulfated	Native	Desulfated
	A	B	C	D			
	<i>ppm</i>				<i>ppm</i>		
Proton							
H-1	5.343	5.293	5.094	5.050	5.070	5.300	5.053
H-2	4.596	4.596	3.950	3.887	3.950	4.570	3.917
H-3	4.268	4.192	4.126	4.051	4.076	4.200	4.063
H-4	<i>4.054</i>	<i>4.054</i>	<i>3.983</i>	<i>3.972</i>	<i>3.970</i>	<i>4.030</i>	<i>3.962</i>
H-5	4.535–4.590	4.535–5.900	4.535–4.590	4.535–4.590	4.580	4.590	4.590
H-6	1.330–1.430	1.330–1.430	1.330–1.430	1.330–1.430	1.370	1.360	1.356
Carbon							
C-1	101.97	101.60	103.40	103.03	103.50	98.20	99.80
C-2	77.90	77.90	71.30	71.30	71.35	74.50	67.94
C-3	70.97	71.40	70.97	70.97	67.69	66.79	67.94
C-4	<i>83.40</i>	<i>83.40</i>	<i>83.90</i>	<i>83.90</i>	<i>82.75</i>	<i>80.10</i>	<i>79.80</i>
C-5	69.96	69.96	69.96	69.96	70.02	66.98	66.78
C-6	13.00	13.00	13.00	13.00	13.00	14.00	14.00

^a Sulfated fucan I contains four types of α -fucose residues (see Figs. 4A and 5A).

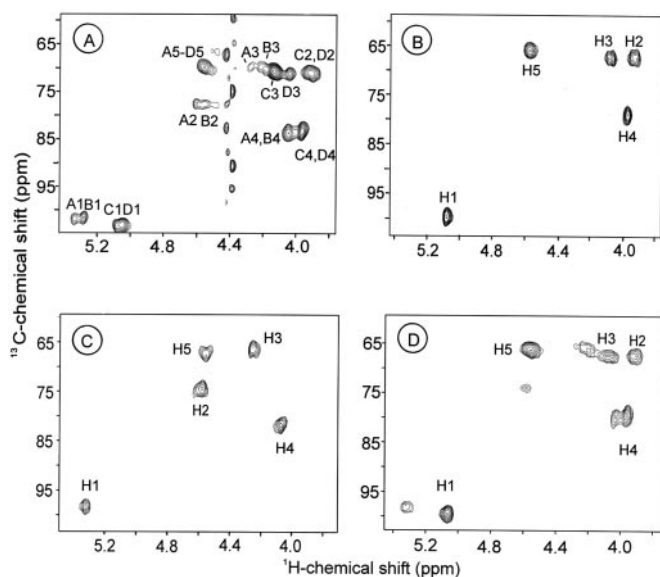


FIG. 5. ¹H/¹³C HMQC spectra of native (A and C) and desulfated (B and D) α -fucan I (A and B) and α -fucan II (C and D) from *S. droebachiensis*. The assignment was based on TOCSY and COSY spectra. The values of chemical shifts in Table III are relative to external trimethylsilylpropionic acid at 0 ppm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

from *S. droebachiensis*, H-1 of residue A shows cross-peaks to H-4 of residue B; H-1 of residue B shows cross-peaks to H-4 of residue C; H-1 of residue C shows cross-peaks to H-3 of residue D; and H-1 of residue D shows cross-peaks to H-2 of residue A. This evidence indicates the sequence and linkage -4-A-1→4-B-1→4-C-1→4-D-1→, as shown in Fig. 7A.

The presence of minor random components in sulfated α -fucan I cannot be ruled out. For example, small amounts of three consecutive 2-*O*-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 (Figs. 4A and 5A) indicate these additional structures cannot account for a substantial proportion of the sulfated fucan structure.

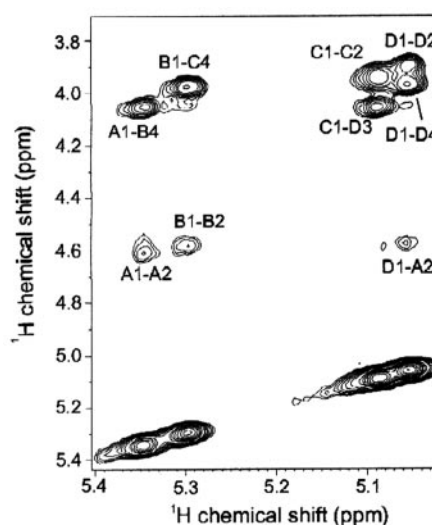


FIG. 6. Expansion from the NOESY spectrum of the sulfated α -fucan I from *S. droebachiensis*. The four fucose residues in the repeating unit are marked A–D as in Fig. 4A. We can observe NOEs from H-1 of each residue to the following ring proton, in particular the sequence-defining NOEs A1–B4, B1–C4, C1–D3, and D1–A2.

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

³ Different samples of desulfated fucan II were used for methylation and NMR analyses. Totally desulfated fucan II was employed for methylation analysis (Table II), whereas a partially desulfated preparation was used for NMR analysis (Figs. 4D and 5D).

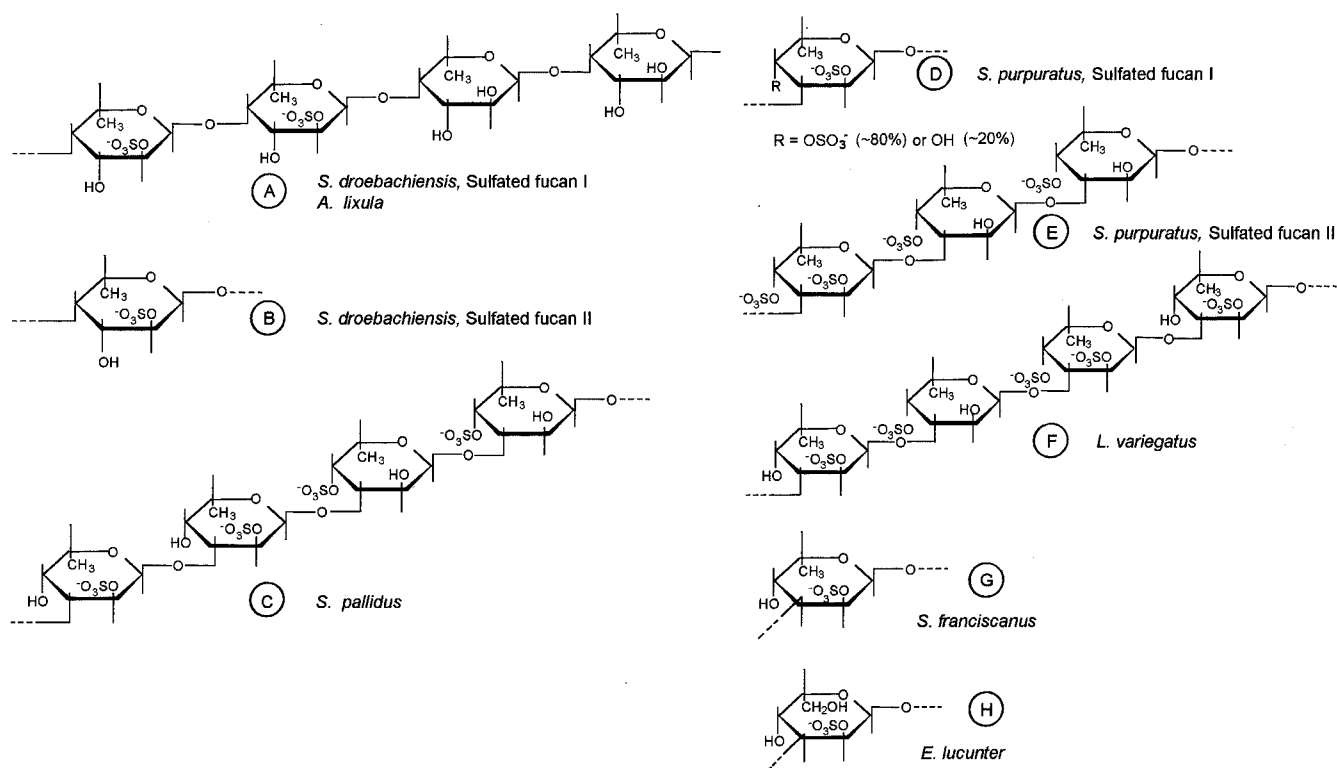


FIG. 7. Structures of sulfated α -L-fucans and sulfated α -L-galactan from sea urchin egg jelly. Shown are eight fully characterized structures of sulfated polysaccharides from the egg jellies of seven species of sea urchins. The specific pattern of sulfation, the position of the glycosidic linkage, and the constituent monosaccharide vary among sulfated polysaccharides from different species (5–7, 27). See “Results and Discussion” for details.

fucan II is totally 2-*O*-sulfated. It contains a single spin system, and alterations upon desulfation are consistent with 2-*O*-sulfation: -0.65 ppm for H-2, -0.14 ppm for H-3; -0.07 ppm for H-3; -6.6 ppm for C-2; and $+1.1$ ppm for C-3 (Figs. 4 (C and D) and 5 (C and D) and Table III).

In conclusion, methylation and NMR analyses indicated that sulfated fucans I and II from *S. droebachiensis* are linear polysaccharides composed of α 1 \rightarrow 4-linked fucopyranose. The two fucans differ in their sulfation pattern. Sulfated fucan I consists mostly of a regular sequence of four residues ([4- α -L-Fucp-2(OSO₃)₂-1 \rightarrow 4- α -L-Fucp-2(OSO₃)₂-1 \rightarrow 4- α -L-Fucp-1 \rightarrow 4- α -L-Fucp-1]_{*n*}), whereas sulfated fucan II is a homopolymer of 4- α -L-Fucp-2(OSO₃)₂-1 units (Fig. 7, A and B). In addition, NMR analyses showed the absence of intermediate fractions between sulfated fucans I and II and confirmed that these two polysaccharides have a well defined repeating unit determined by specific patterns of sulfation.

The Sulfated α -Fucan from S. pallidus Has a 3-Linked Tetrasaccharide Repeating Unit Defined by a Specific Pattern of Sulfation at the 2-O- and 4-O-Positions—The sulfated fucan from *S. pallidus* that eluted from an anion-exchange chromatography column at 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 upon 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 (Figs. 8A and 9A), whereas after desulfation, a single anomeric signal was seen (Figs. 8B and 9B), as already observed for sulfated fucan I from *S. droebachiensis* (Figs. 4 (A and B) and 5 (A and B)). But, in the case of desulfated fucan from *S. pallidus*, a strong downfield shift (~ 8 ppm) of C-3 (Table IV, values shown in italic type), and not of C-4, 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 H-2 of residues A and B (-0.50 ppm) and H-4 of residues C and D (-0.70 ppm) (Fig. 10A and Table IV) indicate that two of the four residues are 2-*O*-sulfated and that the other two are 4-*O*-sulfated. Minor structural components, which may occur in this sulfated α -fucan (such as those indicated by arrows in Fig. 8A), do not account for $>5\%$ of the total signals in the anomeric region based on integration of the peaks in this region of the ¹H spectrum. In addition, the proportions of these minor components (but not those of the A–D spin systems) vary among different preparations of sulfated α -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 spectrum. Although only one inter-residue NOE could be unambiguously identified in the NOESY spectrum (Fig. 10B), it was enough to confirm the proposed structure. Thus, it was possible to identify NOEs from H-1 of residue A to H-4 of residue D, whereas H-1 of residue B, C, or D does not have any inter-residue NOEs. These NOEs are in agreement with the repeat-

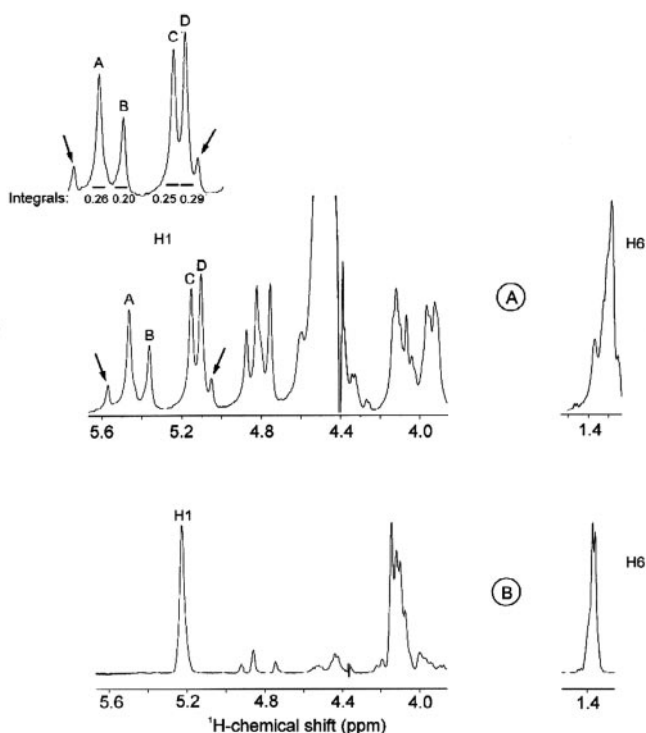


FIG. 8. ^1H one-dimensional NMR spectra at 600 MHz of the native (A) and desulfated (B) α -fucans from *S. pallidus*. Polysaccharide samples and conditions for NMR spectra were as described in the legend of Fig. 4. Expansion of the 5.0–5.6 ppm region of the spectrum is shown in the inset of A. The integrals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

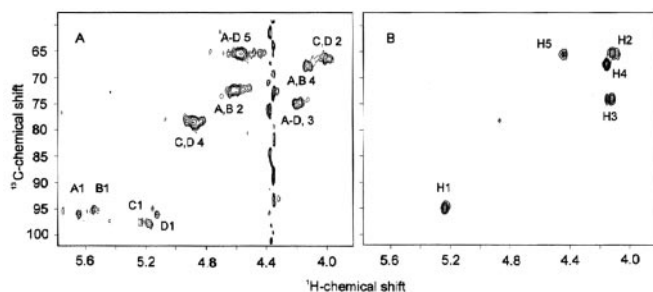


FIG. 9. $^1\text{H}/^{13}\text{C}$ HMQC spectra of native (A) and desulfated (B) α -fucans from *S. pallidus*. The assignment was based on TOCSY and COSY spectra, and the values of chemical shifts are in Table IV. See the legend of Fig. 5 for additional information about the spectra. The anomeric signals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

ing unit of this sulfated fucan as -B-A-D-C- (Fig. 7C).

Overall, the NMR analyses indicated that the sulfated fucan from *S. pallidus* is composed mostly of a regular sequence of four residues, as follows: $[3\text{-}\alpha\text{-L-Fucp-2}(\text{OSO}_3)\text{-1}\rightarrow 3\text{-}\alpha\text{-L-Fucp-2}(\text{OSO}_3)\text{-1}\rightarrow 3\text{-}\alpha\text{-L-Fucp-4}(\text{OSO}_3)\text{-1}\rightarrow 3\text{-}\alpha\text{-L-Fucp-4}(\text{OSO}_3)\text{-1}]_n$ (Fig. 7C). As in the case of sulfated α -fucan I from *S. droebachiensis*, we cannot rule out the occurrence of minor random components 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 of Variants of Sulfated α -L-Fucans from Sea Urchin Egg Jelly—A variety of sulfated fucans have been 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 com-

plex, 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 sea urchin egg jelly. 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 variegatus* (5, 27) have 3-linked sulfated α -L-fucans with tetrasaccharide repeating units that differ in specific patterns of sulfation (Fig. 7, C 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. 7, D and E, respectively) (6). *S. droebachiensis* (sulfated fucan II), *Strongylocentrotus franciscanus* (7), and *Echinometra lucunter* (5) have polysaccharides with a single 2-O-sulfated monosaccharide unit that differ either in the position of their glycosidic linkage or in their constituent monosaccharide (Fig. 7, B, G, and H, respectively). *S. droebachiensis* (sulfated fucan II) and *S. franciscanus* contain 4- and 3-linked α -L-fucopyranose, respectively, whereas *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 sea urchin egg jelly 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 that it has no further role in embryo development. These polysaccharides are species-specific as inducers of the sperm acrosome reaction and may represent one of the barriers that prevent interspecific fertilization.

We have now fully characterized eight sulfated polysaccharides from the egg jellies of seven species of sea urchins (Fig. 7). We can now formulate questions such as follows. What are the common structural features among these polysaccharides? Can we identify the structures that confer finer specificity of recognition in 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 α -fucose units (7).

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

TABLE IV

Proton and carbon chemical shifts for residues of α -fucose in native and chemical desulfated fucans from *S. pallidus*

The spectra were recorded at 600 MHz in 99.9% D₂O. Chemical shifts are relative to external trimethylsilylpropionic acid at 0 ppm for ¹H and to methanol for ¹³C. Values in boldface type indicate positions bearing a sulfate ester, and those in italic type indicate glycosylated positions.

	Native ^a				Desulfated
	A	B	C	D	
	<i>ppm</i>				<i>ppm</i>
Proton					
H-1	5.522	5.430	5.200	5.120	5.230
H-2	4.594	4.602	4.016	4.062	4.090
H-3	<i>4.180</i>	<i>4.180</i>	<i>4.194</i>	<i>4.199</i>	<i>4.090</i>
H-4	4.120	4.120	4.853	4.894	4.130
H-5	4.469–4.560	4.469–4.560	4.469–4.560	4.469–4.560	4.430
H-6	1.322–1.430	1.322–1.430	1.322–1.430	1.322–1.430	1.380
Carbon					
C-1	94.50	97.00	97.20	95.70	94.52
C-2	75.90	75.90	70.05	70.05	65.35
C-3	<i>78.55</i>	<i>78.55</i>	<i>78.55</i>	<i>78.55</i>	<i>74.01</i>
C-4	71.80	71.80	81.80	81.80	67.59
C-5	69.13	69.13	69.13	69.13	65.23
C-6	15.00	15.00	15.00	15.00	13.00

^a The sulfated fucan from *S. pallidus* contains four types of α -fucose residues (see Figs. 8A and 9A).

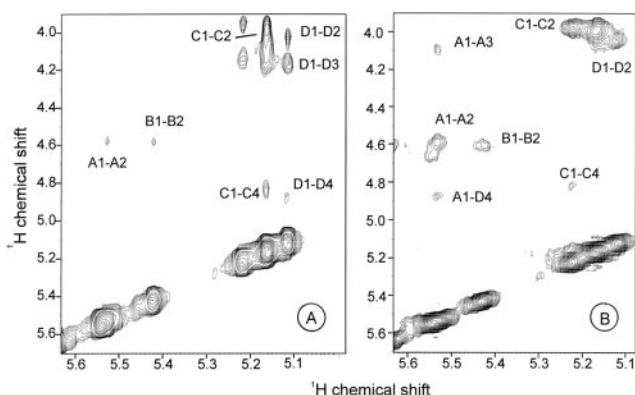


FIG. 10. Expansions of the TOCSY (A) and NOESY (B) spectra of the sulfated fucan from *S. pallidus*. The TOCSY spectrum (A) shows some cross-peaks used in the assignment of the fucose residue, especially positions bearing sulfate esters. The NOESY spectrum (B) shows NOEs, especially the sequence-defining A1–D4. The four fucose residues in the repeating unit are marked A–D as described in the legend of Fig. 8.

fucans enriched in 4-*O*-sulfated residues (7).

The two new species of sea urchins we have now studied allow a more in depth analysis concerning the species specificity of sulfated α -fucans as inducers of the acrosome reaction in echinoderms. The sulfated α -fucans from these species contain two consecutive 2-*O*-sulfated fucose residues, which alternate either with two unsulfated or 2-*O*-sulfated residues (in *S. droebachiensis*) or with two 4-*O*-sulfated fucose units (in *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 repeating unit determines the sperm reactivity.

In our previous studies, we quantified the proportion of sperm that underwent 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 *S. droebachiensis* sperm. We overcame this limitation by measuring fertilization successes among three species of *Strongylocentrotus* (Table V). We were able to identify the contribution of the sperm acrosome reaction to the interspecific fertilization of these species by comparison of 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 that the induction of the 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* in achieving heterospecific fertilization without pre-activated sperm, indicating a slightly lower species specificity (Table V). We cannot determine whether 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 tetrasaccharide repeating unit.

For *S. purpuratus* eggs, we still did not detect fertilization after pre-reaction of the sperm with conspecific egg jelly. Therefore, additional steps of gamete interaction, in addition to 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 references therein).

Overall, the experiments summarized in Table V indicate that the sulfated α -fucans from the egg jellies of *S. pallidus* and *S. droebachiensis* induce the acrosome reaction in homologous (but not heterologous) sperm. This was confirmed by recent assays of acrosomal exocytosis using immunofluorescence microscopy and anti-bindin antibody.⁴ Again, the immunological staining of sperm after incubation with the purified sulfated α -fucans demonstrated that the egg jelly polysaccharides induce the acrosome reaction in homologous (but not heterologous) sperm. This is the major limitation for interspecific fertilization 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 in the cascade of gamete recognition events.

Two Sulfated α -Fucan Isotypes in a Single Species of Sea Urchin—We have 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 and 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

⁴ C. H. Biermann, unpublished data.

TABLE V
Fertilization success of plain sperm and sperm pre-reacted with egg jelly for crosses among three *Strongylocentrotus* species

Sperm	Eggs	Pre-reaction with conspecific egg jelly	Mean % of eggs fertilized ^a	Ratio of fertilization after and before pre-reaction with conspecific egg jelly
<i>S. droebachiensis</i>	<i>S. droebachiensis</i>	-	80	1.06
	<i>S. pallidus</i>	+	85	
	<i>S. purpuratus</i>	-	9	6.67
	<i>S. purpuratus</i>	+	60	
<i>S. pallidus</i>	<i>S. droebachiensis</i>	-	<1	3.67
	<i>S. droebachiensis</i>	+	<1	
	<i>S. pallidus</i>	-	24	1.07
	<i>S. pallidus</i>	+	88	
	<i>S. purpuratus</i>	-	73	
	<i>S. purpuratus</i>	+	78	
	<i>S. purpuratus</i>	-	<1	
	<i>S. purpuratus</i>	+	<1	

^a The values are the percentage of eggs fertilized at a sperm concentration of 200 sperm/ μ l before (-) and after (+) pre-reaction with conspecific egg jelly.

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 -fucan isoforms of *S. droebachiensis* have well defined sulfation patterns and are not a consequence of variable degrees of sulfation (Fig. 7, A and B). The inheritance of such sulfation patterns is unknown. We expect 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.⁵ Of course, we cannot exclude unique metabolic pathways, as reported for the biosynthesis of a sulfated α -L-galactan from ascidians (34, 35). For example, an alternative to explain the presence of either sulfated fucan I or 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 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 repeating structure (Fig. 7A). Our recent experiments (not shown) with immunological staining of *S. droebachiensis* sperm with anti-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.

⁵ In the case of *S. pallidus*, two additional sulfotransferases may be involved in the biosynthesis of the sulfate fucan: one transferase to recognize the two consecutive 2-O-sulfated fucose units and then to sulfate C-4 of the third residue and another transferase to recognize the sulfation pattern of the first three fucose residues and then to sulfate the fourth unit at C-4 to obtain the repeating sequence shown in Fig. 7C.

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