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Isolation of Polysaccharides Sulfated during Early Embryogenesis in *Fucus*[1]

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ABSTRACT

Beginning 10 hours after fertilization, zygotes of *Fucus distichus* L. Powell incorporate ^{35}S into polysaccharides as a sulfate ester of fucose. These sulfated polysaccharides are sequestered in only the rhizoid cell of the two-celled embryo and can serve as a marker of cellular differentiation. Zygotes were pulsed at different times after fertilization with $Na_2{}^{35}SO_4$ to identify and isolate the fucans localized within the region of cytoplasm destined to become the rhizoid cell. Low molecular weight pools of ^{35}S were saturated within 60 minutes, with the greatest incorporation into ethanol-soluble and insoluble fractions occurring with 0.1 mM Na_2SO_4 in the artificial sea water medium. At the time of rhizoid formation, four fucose-containing polysaccharide fractions incorporatd ^{35}S. When each fraction was subjected to diethylaminoethyl chromatography, two components were eluted with KCl that contained over 84% of the fucose and 93% of the ^{35}S of the particular fraction. High-voltage paper electrophoresis of each fraction also resulted in the separation of these two major components. Both components from each of the four fractions behaved identically when separated by diethylaminoethyl chromatography and paper electrophoresis. By comparing the incorporation of ^{35}S into the polysaccharide fractions at 4 and 16 hours after fertilization, the fucan-sulfate components that are localized in the cytoplasm at the time of rhizoid formation were isolated. Although sulfated polysaccharides in brown algae are reported to be very heterogeneous in terms of their sugar composition and complexes with other heteropolymers, we propose that there are two major components that are sulfated during early embryogenesis in *Fucus*. The location of these two sulfated polysaccharides in different chemical fractions may reflect their subcellular localization (*e.g.*, cytoplasmic vesicles or cell walls), or their association with other heteropolymers.

Differentiation is characterized by the appearance of cell-specific products. The rhizoid cell of the two-celled *Fucus* embryo contains a sulfated polysaccharide that can be detected cytochemically in the cytoplasm and in a mucilaginous layer around the cell wall. This polymer cannot be detected in the thallus cell or in the young zygote by cytochemical or autoradiographic techniques (17). Therefore, this sulfated polymer that accumulates in the rhizoid cell can be used as a biochemical marker of differentiation, and *Fucus* utilized as a model system to study cytoplasmic localization, a phenomenon characteristic of many embryos (5, 17).

During early embryogenesis in *Fucus*, exogenous $^{35}SO_4{}^{2-}$ from sea water was incorporated into an acid-soluble fucose polymer at about 10 hr after fertilization. At the same time a sulfated polysaccharide appeared localized by autoradiographic and cytochemical techniques (12, 17). The lack of sulfate incorporation into this fucan fraction before 10 hr did not appear to be a result of permeability barriers to sulfate entry into zygotes, or changes in the intracellular sulfate pool (18). Indirect evidence also indicated that this enzymatic sulfation may be instrumental in localizing the fucan-sulfate in the rhizoid cell (4, 18) via an intracellular electrical field (7).

Although this fucan fraction was identified as a sulfate acceptor, substantial amounts of other heterogeneous sulfated polysaccharides have been recently isolated and characterized from brown algae (1, 2, 8, 13, 14). The extent of the sulfation of these various polymers in relation to cellular differentiation has not been determined in two-celled embryos.

To study the biochemical mechanism operative in the location of the sulfated polysaccharides, and to understand the regulation of the sulfation process, isolation and subsequent characterization of the primary acceptor(s) was required. The purpose of this research was to determine the distribution of ^{35}S as the ester sulfate of fucose in various polysaccharide fractions that have been well characterized in other brown algae, and to isolate the major polysaccharide(s) that are sulfated *in vivo* at the time of cellular differentiation in *Fucus* embryos.

MATERIALS AND METHODS

Receptacles of *Fucus distichus* L. Powell were collected from Yaquina Head in Newport, Ore. Zygotes were obtained from the receptacles by previously described techniques, washed in artificial sea water lacking inorganic sulfate, and grown at 15 C in light (17).

For sulfate uptake studies, equal amounts of synchronously developing zygotes were pipetted into Petri dishes containing a final volume of 5 ml in the presence of 0 mM, 0.1 mM 1.0 mM, or 10.0 mM carrier Na_2SO_4. At 4 or 12 hr after fertilization, $Na_2{}^{35}SO_4$ (1 μCi/ml) was added for various times up to 60 min. At the end of the labeling period, zygotes were washed three times with ASW[2] containing the same concentration of carrier Na_2SO_4 in which they were grown. The washed zygotes were homogenized with 90 μm glass beads (Sigma) in a sintered glass Duall homogenizer containing cold 80% (v/v) ethanol. The homogenate was centrifuged (10,000*g*/10 min at 4 C), the pellet resuspended, and washed three additional times in 80% ethanol. The supernatants were com-

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[2] Abbreviation: ASW: artificial sea water.

bined and analyzed (80% ethanol-soluble). The residue (80% ethanol-insoluble) was further extracted with 0.2 N HCl. The extract was precipitated with ethanol according to previously described procedures (18) to yield a fucan fraction ("fucoidan"), and a fraction which was acid-insoluble.

A complete distribution of ^{35}S into various polysaccharide fractions was obtained by labeling zygotes between 12 and 16 hr after fertilization with $Na_2{}^{35}SO_4$ (0.5 μCi/ml), followed by extraction of the zygotes according to the procedures of Mian and Percival (13). Zygotes were homogenized as above in 80% ethanol, centrifuged, resuspended in 2 ml of 37% formaldehyde (Sigma), and allowed to stand overnight at room temperature for polymerization of phenolics. The residue was then sequentially extracted by stirring in the following solutions (6 ml/g): 2% (w/v) aqueous $CaCl_2$ for 1 hr at room temperature and 1 hr at 55 C; 0.2 N HCl (pH 2) for 1 hr at 55 C, and 3% (w/v) aqueous Na_2CO_3 for 1 hr at 55 C. Each extraction was repeated with fresh solution until the radioactivity in the extracts was at background. The fractions extracted with $CaCl_2$ (fraction A) and dilute acid (fraction B) were dialyzed overnight at 4 C against deionized H_2O. The solutions retained within the dialysis membranes were made 95% with respect to ethanol and the resulting precipitates were collected by centrifugation.

The material solubilized with 3% Na_2CO_3 was made 95% in ethanol, the resulting precipitate collected, redissolved in a minimal volume of H_2O and dialyzed exhaustively against deionized H_2O at 4 C. The solution retained within the dialysis membrane was made 0.1 M in $CaCl_2$ and allowed to stand overnight at 4 C to remove alginic acid. The suspension was centrifuged, and the supernatant (fraction C) and pellet (fraction D) were separated. The supernatant was dialyzed against deionized H_2O and the solution retained within the dialysis membrane was taken to dryness on a rotary evaporator. Each of the fractions obtained was dissolved in a minimal volume of deionized H_2O for subsequent analyses.

A portion of each of the fractions obtained was applied to a cellulose (DE-52, microgranular, Whatman) ion exchange column (1 × 15 cm) which had been equilibrated with 0.5 M KCl (5 bed volumes). The column was then eluted with deionized H_2O (1.5 bed volumes), followed by a linear gradient of KCl (0.1 M–2.0 M). Fractions containing 0.5 ml were collected and monitored for radioactivity and fucose content.

High voltage electrophoresis (E-C Apparatus Corp.) was carried out at 0 C in 50 mM phosphate buffer pH 7.0. Samples (200 μl) were applied to Whatman No. 3MM paper and a constant voltage of 1500 v (38 v/cm) was applied for 1 hr. The electrophoretogram was cut into 2-cm sections for determination of radioactivity.

$Na_2{}^{35}SO_4$ (New England Nuclear) had a specific radioactivity of 698 mCi/mmole. Aliquots (50–100 μl) of the various radioactive fractions were applied to Whatman No. 3MM filter discs and air dried. The sample filters were placed in 5 ml of Omnifluor (New England Nuclear) and counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 2425) which operated at 87% efficiency for ^{35}S. All cpm reported in this study were adjusted for background and corrected for quenching by use of an external standard. Sections from electrophoretograms and paper chromatograms were counted in a similar fashion.

Fucose concentration was determined by the cysteine reaction (6). Unpublished observations of ours indicated this assay was specific for fucose; glucose and uronic acids did not interfere. Protein content was estimated by the method of Lowry *et al.* (11). The presence of ester-sulfate was determined by release of ^{35}S from various fractions by hydrolysis with acidic methanol (0.09 N HCl in methanol). Treatment of the same size samples with methanol served as a control.

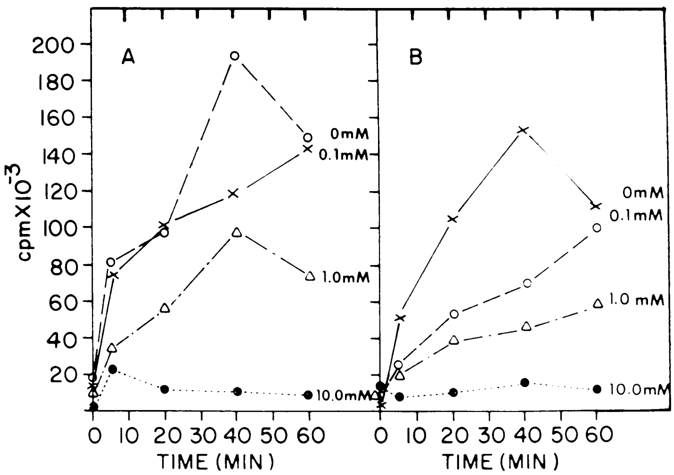

FIG. 1. Time course of $Na_2{}^{35}SO_4$ uptake into the 80% ethanol-soluble fraction of 4-hr zygotes (A) and 12-hr zygotes (B) grown in ASW with various concentrations of Na_2SO_4 added. Each culture compared at 4 or 12 hr had the identical number of zygotes.

Table I. *Distribution of ^{35}S in Various Fractions*

The 12-hr zygotes were labeled for 60 min in the presence of various concentrations of Na_2SO_4.

Na_2SO_4	Radioactivity in Various Fractions			
	80% Ethanol-soluble	80% Ethanol-insoluble	0.2 N HCl-insoluble	0.2 N HCl-soluble
mM		*cpm × 10^{-3}*		
0.0	111	747	624	66
0.1	101	827	756	58
1.0	59	298	229	30
10.0	12	30	19	4

RESULTS AND DISCUSSION

To determine the optimal conditions for uptake of ^{35}S into single cells, and the subsequent incorporation into polymers, $Na_2{}^{35}SO_4$ was added to populations of 4- and 12-hr-old zygotes for time periods of up to 60 min. The amount of labeled low-mol wt compounds and free $^{35}SO_4{}^{2-}$ (80% ethanol-soluble fraction) reached a maximum within 40 to 60 min (Fig. 1). Incubations of 120 min did not appreciably increase the amount of ^{35}S in this soluble fraction. The maximum incorporation was attained with 0 to 0.1 mM Na_2SO_4 in the medium, and there was no significant difference between the 4- and 12-hr cultures (Fig. 1).

Table I indicates that during a 60-min pulse of 12-hr zygotes, 0.1 mM Na_2SO_4 was also optimal for incorporation of ^{35}S into polymers (80% ethanol-insoluble fraction). The same concentration of Na_2SO_4 in sea water was found to be optimal for the incorporation of ^{35}S into carrageenan, a sulfated polysaccharide from the red alga *Chondrus crispus* (10). Unlike the ethanol-soluble compounds, the ethanol-insoluble fraction from *Fucus* still exhibited the initial rate of ^{35}S incorporation after 60 min. Extraction of this polymer fraction with 0.2 N HCl solubilized a fucan-sulfate, classically referred to as fucoidan (15), but released only 6% of the total radioactivity found in the polymer fraction (Table I).

In view of these data and more recent chemical analyses of heteropolysaccharides containing sulfate from several other brown algae (2, 13, 14), we labeled zygotes with Na_2SO_4 for 4 hr during rhizoid cell differentiation (12–16 hr after fertilization) to determine the distribution of ^{35}S-labeled fucans in various well defined fractions (13, 14). It is clear from Table II that the greatest fucose concentration is found in the Na_2CO_3-soluble fraction (fraction C), and that 73% of the ^{35}S incorporated into polymer accumulated in this same fraction.

To determine if these fractions isolated from zygotes at 16 hr were comprised of a mixture of different sulfated fucans, each one was subjected to DEAE column chromatography, and the eluates monitored for ^{35}S and fucose. Fractions A, B, and C gave identical profiles when eluted from the column with increasing concentrations of KCl (0.1 M–2.0 M). The profile of fraction of C given in Figure 2 is typical of each fraction. Two major components, eluting between 0.15 M to 0.35 M (II) and 0.35 M to 0.55 M (III), were isolated and represented over 93% of the total ^{35}S and 84% of the total fucose content of each fraction that was eluted by KCl from the DEAE column (Table III). Component III of the $CaCl_2$ extract (fraction A) was found in very small amounts compared to other fractions. Protein could not be detected in either component, and the presence of ester-sulfate was indicated by the lability of the ^{35}S to acidic-methanol hydrolysis (Table IV). It has been

Table II. *Distribution of ^{35}S and Fucose into Various Polysaccharide Fractions of Zygotes*

Fractionation was performed with 16-hr zygotes after a 4-hr pulse with $Na_2{}^{35}SO_4$.

Fraction	Total ^{35}S	Fucose
	cpm × 10^{-5}	*µg*
A	0.94	280
B	0.24	31
C	14.00	557
D	4.00	138

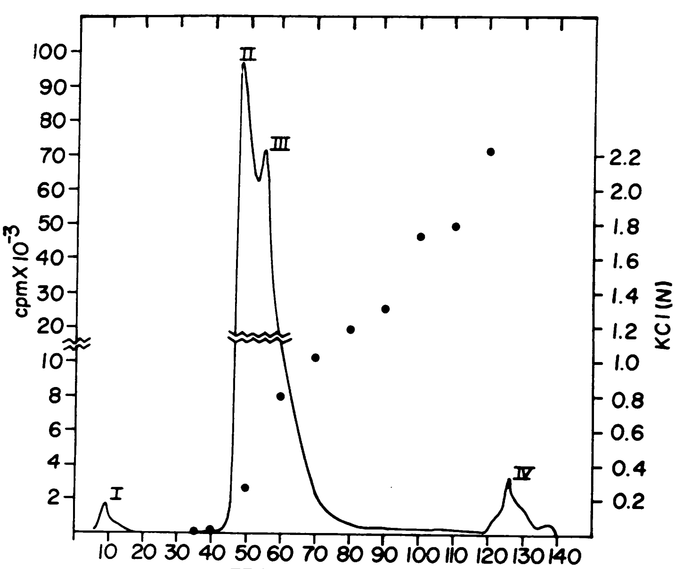

FIG. 2. Ion exchange chromatography of the Na_2CO_3-soluble fraction (fraction C). A Whatman DE-52 cellulose column (1 × 15 cm) was equilibrated in 0.5 M KCl and eluted with deionized H_2O (fractions 1–32), followed by a linear KCl gradient (0.1 M–2.0 M) (fractions 33–140). cpm × 10^{-3} (——); KCl (N) (···).

Table III. *Distribution of ^{35}S and Fucose of Different Polysaccharide Fractions into Two Components Separated by DEAE-cellulose Chromatography*

Fraction	Components			
	II	III	II	III
	% total ^{35}S[1]		*% total fucose*[1]	
A	70.2	27.8	59.0	39.0
B	22.6	71.2	45.0	52.9
C	49.1	47.9	32.1	52.0

[1] % of total recovered from salt gradient (0.1 M–2.0 M KCl). In all three fractions, greater than 80% of the total fucose and 90% of the total ^{35}S applied to the column was recovered from the salt gradient.

Table IV. *Susceptibility of Four Components of Na_2CO_3-soluble Fucan Fraction (C) Resolved by DEAE-chromatography to Ester-Sulfate Hydrolysis Using Acidic Methanol*

Component	Total ^{35}S	Total Fucose	Radioactivity Removed With	
			Acidic methanol	Methanol
	cpm × 10^{-4}	*µg*	%	
I	0.52	23.4	66	16
II	43.1	135.1	82	0
III	40.7	219.6	86	0
IV	2.0	43.1	49	0

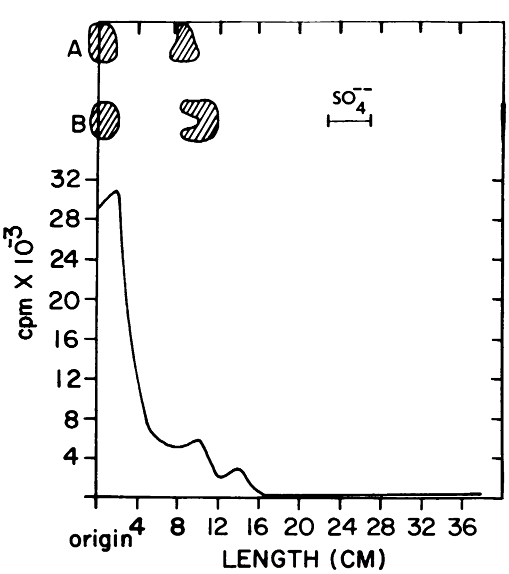

FIG. 3. Electrophoretic separation (50 mM phosphate buffer, pH 7.0) of the ^{35}S-labeled Na_2CO_3-soluble fraction (fraction C) on Whatman 3 MM paper when monitored for radioactivity (——) and alkaline silver-nitrate staining (A and B). A: commercial fucoidan; B: fraction C. Migration of $^{35}SO_4{}^{2-}$ toward the anode is used as a reference.

demonstrated that the maximum amount of ester-sulfate removed from fucoidan standards using this procedure is 80 to 85%, and following this treatment, no sulfate can be detected in the treated fucan by a barium sulfate precipitation procedure (3). No additional components were demonstrated when components II and III were further purified by rechromatography on DEAE using a linear gradient between 0.1 and 1.0 M KCl.

These purified sulfated polysaccharides were then subjected to pH 7 paper electrophoresis. The pattern of ^{35}S distribution upon electrophoresis was matched by alkaline silver-nitrate positive components at the same locations (Fig. 3). Purified component II remained at the origin, while III was separated into two components, IIIa at 8 cm, and IIIb at 16 cm from the origin (Fig. 4). When subjected to electrophoresis commercial fucoidan (K & K Laboratories) exhibited alkaline silver-nitrate positive components at similar locations. Paper chromatography, according to Suzuki and Strominger (19), did not separate II from III; all of the radioactivity and alkaline silver-nitrate staining material remained at the origin.

Mian and Percival (13) reported the isolation of three sulfated polysaccharides by similar DEAE chromatographic methods from the combined aqueous $CaCl_2$ and acid extracts of *Himanthalia lorea, Bifurcaria bifurcata,* and *Padina pavonia.* These polymers were eluted with 0.3 M, 0.5 M, and 1.0 M KCl. Components II and III, separated from fractions A, B, and C of *Fucus* embryos, probably represent polysaccharides similar to those eluted at 0.3 M and 0.5 M KCl from *H. lorea, B. bifurcata,* and *P. pavonia.*

In order to distinguish the fractions(s) and component(s) which are localized within the cytoplasm at the time of differentiation, we pulse-labeled zygotes with $Na_2{}^{35}SO_4$ at two different times after fertilization. The fucan of importance during this localization can be characterized in terms of the time after fertilization it incorporates ^{35}S as an ester, and the amount of sulfate in the polymer at the time of sulfation. Quatrano and Crayton (18) found that the fucan which is localized in the cytoplasm of the rhizoid cell was sulfated only after 10 hr and maximally at 16 to 18 hr after fertilization. Prior to 10 hr this fucan had little detectable sulfate. Although sulfated fucans have been detected in the new cell wall deposited within the first 6 hr after fertilization (9), these are randomly deposited throughout the wall and not localized within the cytoplasm.

Developing zygotes were pulsed for 60 min at the time of

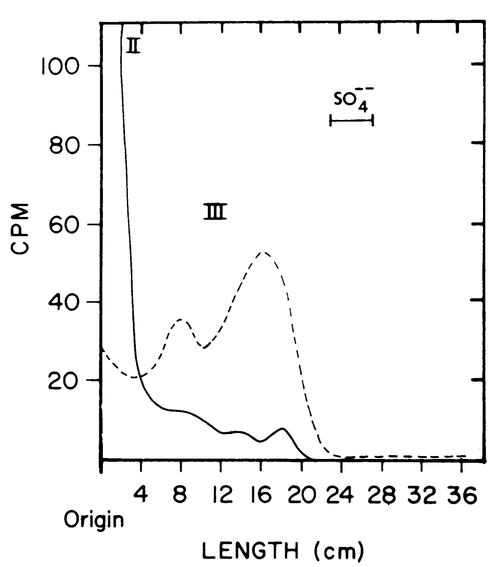

FIG. 4. Electrophoretic separation (50 mM phosphate buffer, pH 7.0) of component II (——) and component III (-----) from Na_2CO_3-soluble fraction (fraction C) after purification by rechromatography on DEAE-cellulose.

Table V. *Distribution of ^{35}S in Various Polysaccharide Fractions*

The polysaccharides were extracted from 4- and 16-hr zygotes following a 60-min pulse with $Na_2{}^{35}SO_4$. Each population of zygotes at 4 and 16 hr had identical cell numbers.

Fraction	Radioactivity	
	4 hr	16 hr
	cpm × 10^{-3}	
A	34.7	176.4
B	26.1	209.4
C	635.9	481.3

cell wall deposition (4 hr) and rhizoid initiation (16 hr), and fractionated as above. Fractions A and B corresponded to the polysaccharide fractions which initially become sulfated during rhizoid initiation (Table V). Incorporation of ^{35}S increased 5- to 8-fold in fractions A and B at 16 hr compared to 4 hr. These polysaccharide fractions have been shown to have a low sulfate content prior to rhizoid initiation (18). In contrast, fraction C had a relatively high sulfate content, 20.5% by weight, when measured at 4 hr, a time prior to rhizoid initiation. Using the same method, mature fronds and commercial fucoidan had values of 28% and 32%, respectively. In addition, fraction C incorporated ^{35}S at 4 hr as well as at 16 hr, and showed a decrease in sulfate incorporation at 16 hr compared to 4 hr (Table V). Fraction C probably corresponds in large part to those sulfated polysaccharides deposited in the new cell wall at 4 hr (9). Still, considerable amounts of ^{35}S were accumulated in fraction C during rhizoid initiation (Table V).

Unsulfated fucans in the cytoplasm that are sulfated at the time of this 60-min pulse are localized in the rhizoid cytoplasm, mostly sequestered in Golgi-derived vesicles (16). As stated above, these sulfated polysaccharides probably correspond to those isolated in fractions A and B. Cytochemical and autoradiographic data (12, 16) are consistent with the interpretation that these sulfated polysaccharides then migrate into the developing rhizoid and are deposited into the newly emerging cell wall to form the mucilaginous cap. Perhaps the polysaccharides sulfated at 16 hr that accumulate in fraction C represent not only those components sulfated in the emerging rhizoid cell wall (similar to the events at 4 hr during wall deposition), but also the fucans sulfated in the cytoplasm (fractions A and B) which are subsequently incorporated into the mucilaginous cap.

Separation by DEAE chromatography and paper electrophoresis of the fucan-sulfate components in fractions A, B, and C that were sulfated *in vivo* at 16 hr revealed two similar components in all fractions. We propose that the release of these two similar sulfated polysaccharides by different extraction procedures may reflect their subcellular localization (*e.g.,* cytoplasmic vesicles or cell walls), or their association with other heteropolymers. Short pulse-labeling experiments with $Na_2{}^{35}SO_4$ indicated that these components in fractions A and B represent those that are localized within the cytoplasm at the time of differentiation.

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