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Fluorescent derivatization of polysaccharides and carbohydrate-containing biopolymers for measurement of enzyme activities in complex media

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Abstract

Fluorescence derivatization provides a means of tracing the dynamics of polysaccharides even in the presence of high concentrations of other organic compounds or salts. A method of labeling polysaccharides with fluoresceinamine was extended to polysaccharides of a wide range of chemical composition, and alternative means of preparation were established for polysaccharides not initially amenable to column chromatography. The polysaccharides were activated with cyanogen bromide, coupled to fluoresceinamine, and separated from unreacted fluorophore via gel filtration chromatography or dialysis. Since the resulting derivatized polysaccharides proved to be stable to further physical and chemical manipulation, methods were also developed for re-activation and labeling with a second fluorophore, as well as for tethering the labeled polysaccharides to agarose beads. As an example of the application of this approach, five distinct fluorescently-labeled polysaccharides (pullulan, laminarin, xylan, chondroitin sulfate, and alginic acid) were used to investigate the activities and structural specificities of extracellular enzymes produced in situ by marine microbial communities, providing a means of measuring specifically the activities of endo-acting extracellular enzymes and avoiding use of low molecular mass substrate proxies. These labeled polysaccharides could be used to explore the dynamics of polysaccharides in other types of complex media, as well as to investigate the activities and specificities of endo-acting enzymes in other systems.

Keywords: Derivatization, LC; Enzyme activity; Polysaccharides; Biopolymers; Carbohydrates

1. Introduction

Polysaccharides and carbohydrate-containing biopolymers play critical roles in a wide range of biological, biochemical, and physical processes, including cellular metabolism, signaling, and adhesion. In marine environments, carbohydrates are important on scales ranging from sub-cellular to global. Carbohydrates constitute 20–40% of the biomass of phytoplankton [1], the base of the marine food chain. Carbohydrates also comprise an estimated 25–50% of dissolved organic carbon (DOC) in seawater [2], the largest active reservoir of organic carbon in the marine environment [3], and thus are important in the global carbon cycle.

Determining the presence and dynamics of these molecules in seawater, sediments, and other complex media, however, presents significant analytical challenges, since they lack spectroscopic or physical characteristics that would enable them to be easily detected in or extracted from most media. For specific types of experimental questions, however,

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fluorescent derivatization can be a fruitful approach. Derivatization of polysaccharides with appropriate fluorophores yields compounds that can be sensitively detected even against high background concentrations of other organic compounds and/or salts.

The fluorescent derivatization of oligo- and polysaccharides has been carried out via a number of methods, including labeling the reducing end of an oligosaccharide with 2-amino pyridine [4]. More recently, Bigge et al. [5] investigated the use of 2-amino benzamide and 2-anthranilic acid in reductive labeling of complex oligosaccharides derived from glycoproteins. Increasing attention to the biochemical role of the glyco- portion of glycopeptides, as well as a need for more specific structural determination of complex mixtures of oligosaccharides has helped focus interest in the development of a wider range of labeling methods and separation techniques for mono- and oligosaccharides. Capillary electrophoresis separation of tetramethylrhodamine [6] and 2-aminobenzoic acid derivatized oligosaccharides [7] have recently been reported, as has a mild method for *N*-substituted hydroxylamine tagging of the reducing end of sugars, which are then suitable for HPLC and mass spectral analyses [8]. These methods, however, are suited only to low molecular mass carbohydrates or to polysaccharides that have been enzymatically or chemically cleaved to low molecular mass products. Fluorescent labeling of polysaccharides has received relatively less attention, although labeling of dextrans has been accomplished using fluoresceinyltriazine to yield thiocarbamoyl linkages [9]. An alternative approach was presented by Glabe et al. [10], who fluorescently-labeled polysaccharides by first activating the polysaccharides directly with cyanogen bromide, followed by coupling at room temperature to fluoresceinamine. This method yielded stable derivatives, and was successfully used with sulfated as well as with neutral polysaccharides.

This method was adapted to a new application, investigation and characterization of the activities and structural specificities of extracellular enzymes that are produced in situ by microbial communities in complex media such as seawater and marine sediments (e.g. Refs. [11–14]). The microbes comprising communities in natural habitats are generally not amenable to isolation; fewer than 1% of extant microbes are estimated to have been obtained in pure cultures [15]. Even among cultured isolates, furthermore, the ability to utilize well-characterized, complex substrates such as specific polysaccharides or carbohydrate-containing biopolymers is seldom investigated. The structural specificity and activity of microbial extracellular enzymes are crucial to heterotrophic microorganisms, however, because they require extracellular enzymes to hydrolyze biopolymers to sizes sufficiently small (ca. 600 Da [16]) for transport across the membrane. The activities of heterotrophic microbial communities and their extracellular enzymes in turn have a major impact on the global carbon cycle, since a high fraction of phytoplankton-produced organic carbon is ultimately cycled by microbes [17]. Fluorescently-labeled polysaccharides can be used to assess and measure extracellular enzyme activities in samples without the need for isolation of specific microbes or their enzymes.

Fluorescently-labeled polysaccharides furthermore provide the means to measure the activities of endoactive enzymes, the enzymes that are responsible for cleavage of a polysaccharide mid-chain. Most commercially available enzyme substrates consist of a fluorophore attached to a mono- or disaccharide; such substrates are unlikely to fit the active sites of endo enzymes, which often contain distinct domains for substrate binding and cleavage [18]. In addition, the activities detected and kinetic parameters determined with such substrate proxies often differ substantially from those determined with the actual polymers [19]. Moreover, only a limited range of carbohydrates is commercially available as proxies to measure enzyme activities. The current work demonstrates that a wide range of polysaccharide substrates can be fluorescently-labeled in order to investigate the activities of specific enzymes as well as to trace the dynamics of well-characterized polysaccharides in natural systems.

This study reports results of ongoing improvements in labeling procedures to produce fluorescently-labeled polysaccharides, plus modifications and new developments enabling an expanded range of polysaccharides and carbohydrate-containing biopolymers to be labeled with fluorophores. The approach of Glabe et al. [10] has been extended to a wider range of polysaccharides, and alternative

means of preparation and synthesis have been explored for polysaccharide substrates initially not amenable to column chromatography. This study shows that the derivatized polysaccharides are stable to a variety of physical conditions as well as further chemical manipulation, enabling them to be labeled with multiple types of fluorophores, and to be tethered to agarose beads. The fluorescently-labeled polysaccharides described here could potentially be applied in a wide range of investigations beyond the boundaries of marine systems.

2. Materials and methods

2.1. Chemicals and materials

Polysaccharides were obtained from Sigma (pullulan, laminarin, maltoheptaose) or Fluka (xylan, fucoidan, chondroitin sulfate, arabinogalactan, alginic acid [sodium salt]). Sodium hydroxide, sodium phosphate (NaH_2PO_4 and Na_2HPO_4), sodium chloride, sodium borate, hydrochloric acid, and cyanogen bromide were purchased from Fluka. Fluoresceinamine (FLA; isomer II), Sephadex gels (G-10, G-25, G-50, and G-75) as well as fluorescein isothiocyanate (FITC) dextrans (150 kDa, 70 kDa, 10 kDa, 4 kDa, FITC-glucose) used to standardize the Sephadex gel columns were obtained from Sigma. Epoxy-activated agarose beads (with a ligand of 1,4-bis (2:3 epoxypropoxy)butane) were also obtained from Sigma. Spectra/Por dialysis membranes (5000 molecular mass cut-off) were obtained from Fisher, and C_{18} Sep-Paks from Waters. Texas Red™ sulfonyl cadaverine (TR) was purchased from Molecular Probes. Milli-Q water was obtained from a Millipore ultrapure water filtration unit.

Borate buffer (0.2 *M*) was prepared by mixing $Na_2B_4O_7{\cdot}10H_2O$ in 1700 ml Milli-Q water on a magnetic stirrer. Over ca. 2 h, several-milliliter portions of 6 *M* HCl were added to the solution, to gradually dissolve borate. Solution pH was adjusted to 8.0 and total volume to 2 l. Phosphate buffer was prepared by dissolving NaCl (100 m*M*), $NaH_2PO_4{\cdot}2H_2O$ (3 m*M*), and $Na_2HPO_4{\cdot}2H_2O$ (47 m*M*) in 1950 ml Milli-Q water, and then adjusting the solution (with NaOH or HCl) to a final pH 8.0 and volume of 2 l. Sephadex gels used for gel filtration chromatography columns were swollen in the appropriate buffer for a minimum of 6 h, and then repeatedly resuspended and let settle, pouring off the overlying buffer in order to remove any fines from the gels prior to pouring columns.

2.2. Fluorescent labeling of substrates

2.2.1. Activation and separation of highly soluble substrates

Activation and labeling of highly soluble polysaccharides (pullulan, laminarin, fucoidan, chondroitin sulfate, arabinogalactan) was carried out as described in Ref. [11], except that initial masses of polysaccharides and volumes of Milli-Q water were doubled to 40 mg and 2.0 ml, respectively, and activation was carried out with triple the mass (30 mg) of CNBr dissolved in 350 μl Milli-Q water.

2.2.2. Activation and separation of substrates requiring initial preparation

Xylan is only moderately soluble, so 40 mg of xylan in 2.5 ml Milli-Q water were sonicated for 5 min, and filtered through a 0.2-μm pore-size surfactant-free cellulose acetate (SFCA) filter before activation as described above.

Alginic acid, a mixed polymer of mannuronic and guluronic acid, is only moderately soluble in basic solution, so an initial solution was prepared at a concentration of 7 mg/ml in 0.25 *M* NaOH. This solution was sonicated, filtered through a 0.2-μm pore-size SCFA filter, and 2.5 ml of the pale yellow solution were injected on a 19×1 cm column of Sephadex G-50 gel in borate buffer. Monitoring the column outflow with a Shimadzu UV–Vis detector (detection wavelength: 490 nm) indicated the presence of several co-eluting fractions of different apparent molecular mass. To increase the quantity of nominally higher molecular mass alginic acid, 182 mg alginic acid was dissolved in 26 ml 0.25 *M* NaOH, yielding a clear yellow solution after sonication. This solution was divided into two equal portions, each of which was dialyzed in Spectra/Por 5000 MWCO membrane versus a total of 8.3 l Milli-Q water (three water changes) over a time course of 144 h. The dialysate was recovered from the membranes and lyophilized, with a recovered mass of 143 mg. Injection on a 29×1 cm column of

Sephadex G-50 gel in borate buffer indicated an increased proportion of material eluted in the void volume of the column. The dialyzed and lyophilized alginic acid was used for all subsequent syntheses, and care was taken to collect only the void volume fraction from the synthesis procedure.

The alginic acid was labeled by dissolving an initial concentration of 12.4 mg alginic acid/ml 0.25 *M* NaOH, and filtering the solution through a 0.2-μm pore-size SFCA filter. The solution was activated by addition to 60 mg CNBr in 650 μl Milli-Q water. Initial pH of the reaction mixture was 10.4, but the pH dropped rapidly with activation of the polysaccharide, and typically a total of 7 (25 μl) portions of 0.25 *M* NaOH were added over a reaction time of 5.5 min to keep the pH above 9.5. The reaction mixture was injected onto a 29×1 cm column of Sephadex G-50 gel in borate buffer at a flow-rate of 1.9 ml/min, and the void volume fraction was collected into a vial containing 6 mg FLA.

Separation of labeled FLA-alginic acid from unreacted fluorophore was achieved by placing the reaction mix into a 5000 MWCO Spectra/Por CE membrane, and dialyzing against a total volume of 10 l Milli-Q water (four water changes). Injection of 250 μl of dialysate onto a Sephadex G-50 column demonstrated that there was no remaining free FLA. The dialysate was lyophilized, and 2.1 mg was dissolved in 1 ml Milli-Q water and filtered.

2.2.3. Double-labeling substrates via reactivation

High molecular mass polysaccharides that can be readily labeled may also be doubly-labeled using a 'shotgun' approach, as previously described [20]. Using this procedure, activation is carried out as described in Section 2.2.1, and the activated polysaccharide is collected in a vial containing two different fluorescent labels.

To ensure that smaller carbohydrates such as maltoheptaose (M_r 1152) are double-labeled, a sequential labeling procedure, rather than a shotgun approach, was developed. The sequential labeling procedure required reactivation of singly labeled substrate: 1800 μl Milli-Q water was added to 200 μl of maltoheptaose initially labeled with FLA, and this starting mixture was activated using CNBr as described in Section 2.2.1. The activated FLA-maltoheptaose solution was injected onto a 9.5×1 cm column of Sephadex G10 gel in borate buffer, and the rapidly-eluting colored band was collected into a vial containing 3 mg of TR (Texas Red) in 400 μl DMF. The reaction mixture was incubated in the dark for 15–18 h.

Since TR tends to interact strongly with the Sephadex gel and oligosaccharides labeled with TR are retarded on column, an alternate separation method for doubly-labeled maltoheptaose was developed using a C_{18} Sep-Pak. The Sep-Pak was washed with 15 ml methanol, followed by 15 ml Milli-Q water, and 200 μl of the reaction mix was applied. The Sep-Pak was then washed sequentially with 2×1 ml Milli-Q water, and then with 1-ml fractions of methanol in water, beginning at 10:90 (%) MeOH/Milli-Q water and increasing the fraction of methanol at each step, collecting each fraction separately.

2.2.4. Attachment of fluorescently labeled substrates to agarose beads

In preparation for tethering labeled polysaccharides to reactive agarose beads, FLA-pullulan was dialyzed in a Spectra/Por 5000 MWCO dialysis membrane to remove phosphate buffer. The dialysate was lyophilized, and 17 mg of lyophilized FLA-pullulan was brought into solution in Milli-Q water (adjusted with 0.25 *M* NaOH to pH 10), mixed, and filtered (0.45-μm pore-size SFCA filter). To prepare the gel, 290 mg of epoxy-activated agarose beads (Sigma; ligand of 1,4-bis (2:3 epoxypropoxy)butane, attached to a matrix of cross-linked 4% beaded agarose) were suspended in pH 10 Milli-Q water. The beads were rinsed by repeated resuspension in pH 10 Milli-Q water followed by gentle centrifugation, with a total rinse volume of 60 ml. The rinsed gel and FLA-pullulan solution were combined in a small vial that continuously rotated in a water bath set to 31 °C. A portion of the beads was removed after 24 h, and the remainder of the beads was left rotating in the water bath for a total of 94 h. After removal from the water bath, the beads were repeatedly rinsed and resuspended alternately in bicarbonate buffer (pH 8.1; prepared by dissolving 0.84 g $NaHCO_3$ and 2.9 g NaCl in 100 ml of Milli-Q water) and phosphate buffer (pH 4.6; prepared by dissolving 1.38 g $NaH_2PO_4.2H_2O$ and 0.58 g NaCl in 100 ml Milli-Q water).

2.3. Chemical characterization of substrates

Carbohydrate concentrations of substrates were measured using the phenol–sulfuric acid method [21], with pullulan, laminarin, xylose, fucose, arabinogalactan, chondroitin sulfate, and alginic acid as standards. Fluoresceinamine concentrations were measured by absorption (490 nm), using a series of FLA solutions in pH 8.0 phosphate buffer as standards. Calculated values assume no change in fluorescence yield upon labeling.

2.4. Measurement of extracellular enzyme activities in complex media

2.4.1. Experimental set-up

Extracellular enzymatic activity can be measured in complex media such as seawater or marine sediments by injecting a fluorescently labeled substrate into intact sediment cores [11], mixing it into homogenized sediments, or adding it to seawater [14]. The sediments or seawater are incubated in the dark at in situ temperature, and subsamples are collected periodically over a time course of incubation (typically ranging from 12 to 144 h for sediments, 48 to 360 h for seawater) in order to monitor the extent of hydrolysis.

The experiments described here were carried out with surface seawater (T: 5.4 °C; salinity: 32‰) and surface sediments collected via Haps-corer (water depth: 212 m; T: 2.3 °C) in September 2001 from Smeerenburgfjorden (Station J: 79° 42.81 N, 44° 05.20 E), a fjord along the west coast of Svalbard, Norway. The equivalent of 175 nmol (monomer concentration) of a single fluorescently labeled polysaccharide (pullulan, laminarin, xylan, chondroitin sulfate, or alginic acid) was added to 50 ml seawater, mixed, and then divided into three replicate incubation vials yielding a concentration of 3.5 μ*M* monomer-equivalent. At each time point, approximately 1.3 ml seawater was removed from each vial, filtered through a 0.2-μm pore-size SFCA filter, and frozen until analysis. For sediment incubations, surface sediments (0–1.5 cm depth) were homogenized and 20-ml portions dispensed into replicate tubes. The equivalent of 3.5 μmol (monomer concentration) of a single substrate was added to each tube, yielding a concentration of 175 μ*M* monomer-equivalent. Triplicate tubes were prepared for each substrate. At each time point, the tubes were centrifuged (2000 rpm, 3–5 min), approximately 1.1 ml of porewater was removed and filtered through a 0.2-μm pore-size SFCA filter, and the porewater sample was stored frozen until analysis.

2.4.2. Analytical system and sample analysis

These samples were analyzed using a chromatographic system consisting of a Shimadzu LC-10AT HPLC pump with pH 8.0 phosphate buffer, flow-rate 1 ml/min. All of the empty columns, flow-injectors, connecting tubing (1.2 mm I.D., Tygon) and fittings were obtained from Bio-Rad. The injector consisted of a three-way valve directly attached to a 14×1 cm flow-adaptor with replaceable frit. Two columns were attached in sequence: a 21×1 cm column of Sephadex G-50 gel, attached via a 7×1 cm flow-adaptor to an 18.5×1 cm column of Sephadex G-75 gel. The G-75 column was poured over a 1-cm base layer of G-25 gel to prevent clogging of the frit at the base of the column. The column outflow went through a Hitachi L-7480 fluorescence detector, set to excitation and emission wavelengths of 490 and 530 nm, respectively. Data were collected and processed using the Knauer EuroChrom 2000 integration package (Knauer, Berlin, Germany) on a Dell XPi laptop computer.

Each sample was individually thawed, and 50–400 μl (porewater) or 600–900 μl (seawater) sample pipeted into a clean vial. Milli-Q water was added to reach a uniform injection volume of 1.1 ml. Note that if an autosampler or narrow-gauge tubing is used between the injector and column, phosphate buffer should be added to the sample instead of Milli-Q water, and the sample should be re-filtered in order to remove precipitated seawater salts prior to injection on the chromatography system. Wide-bore tubing is not affected by the fine-grain precipitates, however, and the only adjustment needed for such systems is regular exchange of the replaceable frit on the Bio-Rad flow adaptors.

The Sephadex GPC columns are calibrated by determining the elution times of FITC dextran standards covering a range of molecular masses. The system described above is a compromise between resolution of a series of standards (>50 kDa, 10 kDa, 4 kDa, monomers, and free fluorophore) and

minimizing per-sample analysis time. Since a single set of experiments can easily yield dozens to hundreds of samples, even the current system (average analysis time per sample: 80 min) frequently requires round-the-clock operation for weeks.

2.4.3. Calculation of enzymatic hydrolysis rates

Hydrolysis rates are calculated by quantifying the changes in molecular mass distribution of a fluorescently labeled polysaccharide as it is enzymatically hydrolyzed to progressively smaller sizes, in effect a comparison between chromatograms from the zero timepoint and after a given interval of sample incubation. Via the FITC dextran standards, the molecular mass range corresponding to a particular elution time is known. The initial molecular mass distribution of a polysaccharide is also known. As previously discussed [11,12,14], the hydrolysis rate can be calculated as the minimum number of hydrolyses which would be required to reduce a polysaccharide from its initial molecular mass to the molecular mass distributions observed after a known incubation time. By convention, carbohydrate hydrolase activities are reported in units of nmol monomer vol^{-1} $time^{-1}$. The 'nmol monomer' term refers to the production of a free reducing end (a free hydroxyl group at the ≠1 carbon position of a monosaccharide) after the bond connecting two monomers is hydrolyzed. This convention is used because many of the techniques for measurement of carbohydrate hydrolase activity actually detect the presence of a free reducing end of a carbohydrate [22].

3. Results

A variety of polysaccharides of different chemical composition were successfully labeled with fluoresceinamine, yielding a series of polysaccharides that are easily detected in complex media. Attachment to polysaccharides shifts the absorption maximum of FLA by approximately 2 nm (from 490 to 492 nm), but does not otherwise detectably change absorption characteristics. The labeling densities (label/monomer) of the polysaccharides differed considerably (Table 1), from a high of 31.4 for pullulan, to a low of 0.15 for alginic acid. The differences in labeling densities obtained in this study are likely due to chemical differences among these polysaccharides. Although labeling densities are affected by solution pH as well as by CNBr concentration [10], the solution conformation of a specific polysaccharide also clearly has an effect. For example, under the same experimental conditions, pullulan is consistently labeled at a higher density than laminarin, although they differ only in linkage type (α(1,4)/α(1,6) for pullulan; β(1,3) for laminarin). Such differences are not surprising, given other differences among polysaccharides differing only in linkage orientation (e.g. the low solubility of cellooligosaccharides, which consist of β(1,4)-linked glucose, relative to pullulan and laminarin).

Lyophilized and redissolved labeled polysaccharides showed no detectable spectroscopic or chromatographic changes relative to initial solutions. Once labeled, the carbohydrates are stable to reactivation: the absorbance spectrum of re-activated FLA-

Table 1
Chemical characterization of polysaccharides labeled with fluoresceinamine (FLA)

Polysaccharide [monomer composition]	Monomer (m*M*)	FLA (μmol)	Labeling density (mmol FLA/mol monomer)
Pullulan [glucose]	20.0	628	31.4
Laminarin [glucose]	19.5	249	12.8
Xylan [xylose]	6.4	58	9.1
Fucoidan [sulfated fucose]	13.0	8.4	0.65
Arabinogalactan [galactose, arabinose]	17.3	45	2.6
Chondroitin sulfate [*n*-acetyl galactoseamine, glucuronic acid]	12.8	18.9	1.5
Alginic acid [mannuronic acid, guluronic acid]	26.7	3.9	0.15

maltoheptaose in borate buffer was indistinguishable from the spectrum of the initial FLA-maltoheptaose, permitting attachment of a second label. Doubly-labeled maltoheptaose was separated from singly-labeled maltoheptaose in the reaction mixture by elution from the Sep-Pak with increasing proportions of methanol in water (Fig. 1). FLA-labeled maltoheptaose eluted in the 10 and 20% MeOH in H_2O fractions, FLA/TR maltoheptaose in the 30 and 40% MeOH fractions, while the TR-labeled maltoheptaose eluted in the 60% MeOH fraction. Stability to lyophilization was also a key to 'tethering' FLA-polysaccharides to reactive agarose beads. Observation of the beads under a fluorescence microscope (excitation and emission wavelengths, 490/530 nm) demonstrated that beads incubated for 24 h were distinctly fluorescent, and those incubated for 94 h appeared to be significantly larger, with a bright 'fuzzy' coat of fluorescent material.

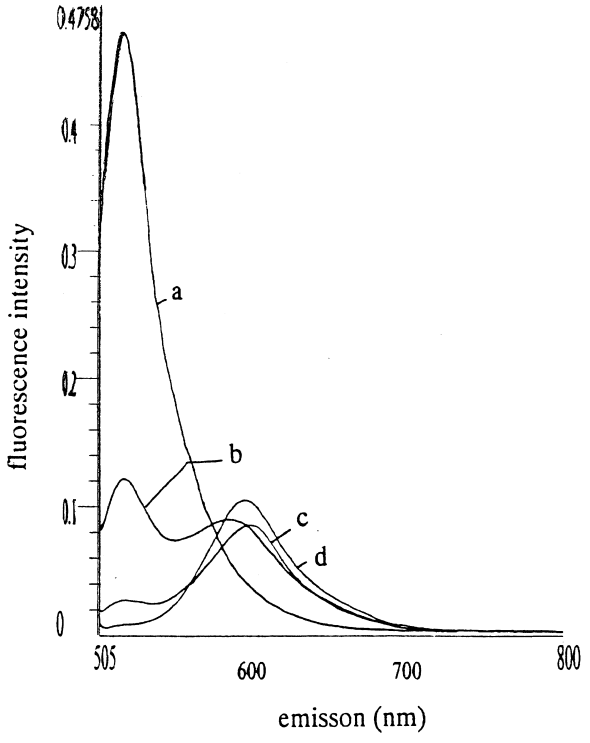

Fig. 1. Fluorescence spectra of fractions eluted from Sep-Pak during synthesis of doubly-labeled FLA/TR-maltoheptaose: 10:20 (%) MeOH in H_2O (a); 30:40 (%) MeOH in H_2O (b); 50% MeOH in H_2O (c); 60% MeOH in H_2O (d).

When added to complex media such as seawater and marine sediments, FLA-polysaccharides were readily detected. FLA-polysaccharide hydrolysis was determined as a change in molecular mass distribution as substrate hydrolysis progressed, showing, for example, that chondroitin sulfate was readily hydrolyzed in both seawater and sediments (Fig. 2). All five polysaccharides were extensively hydrolyzed after 72- to 144-h incubation in homogenized sediments. In seawater, in contrast, there were considerable differences among polysaccharide hydrolysis rates (Fig. 3), and pullulan hydrolysis was not detectable after 216-h incubation. Overall, hydrolysis rates in seawater decreased in the order chondroitin sulfate>laminarin≥xylan>alginic acid≫pullulan. In general, hydrolysis rates calculated for seawater incubations after 216-h incubation were more rapid than at 120 h.

In homogenized sediments, the differences in hydrolysis rates among the five polysaccharides were smaller than in sediments. Hydrolysis rates (on a volume basis) were much more rapid in sediments than in seawater (note difference in units for Fig. 3). Overall, hydrolysis rates in sediments decreased in the order pullulan>laminarin>alginic acid>xylan>chondroitin sulfate, although chondroitin hydrolysis rates more than doubled between 72 and 144 h and were then quite close to the hydrolysis rates of the other polysaccharides. Decreases in hydrolysis rates between 72 and 144 h for pullulan, laminarin, and alginic acid were due to the fact that substrate hydrolysis was substantial at time points prior to 144 h, resulting in little change in molecular mass distribution with further incubation.

4. Discussion

Fluoresceinamine has proven to be a particularly robust and versatile fluorophore for derivatizing polysaccharides that can then be studied under a wide range of conditions. The labeled polysaccharides are stable to repeated freezing/thaw cycles and lyophilization, extended storage at room temperature [11], as well as to incubation temperatures up to 40 °C for 60 h [13]. The FLA fluorophore is particularly appropriate for application in environmental samples because its excitation and emission

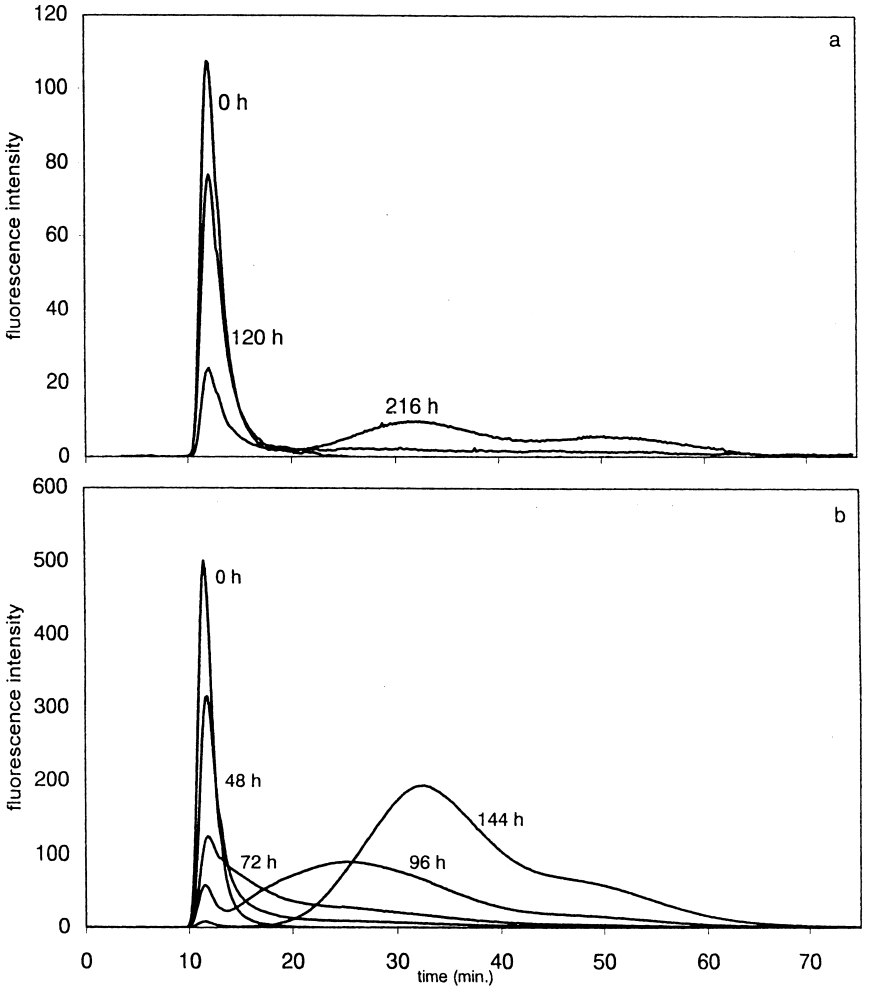

Fig. 2. Elution profiles of fluorescently labeled chondroitin sulfate during hydrolysis in seawater (a) and homogenized sediments (b).

maxima (490/530 nm) are well separated from the excitation and emission maxima (350/450 nm) of chromophoric organic matter dissolved in natural waters [23]. A previous study [20] demonstrated that other fluorophores containing reactive primary amines can also be used to label polysaccharides, although (as with TR) they must often be dissolved in DMF or another water-miscible solvent prior to use. (Note that although TR reacts readily under the conditions tested, its use in environmental samples has been limited by the fact that TR fluorescence—unlike FLA—is apparently rapidly quenched in sediments, although it is detectable in seawater.) The suitability of a specific fluorophore for a given application in complex media must therefore be thoroughly tested.

Polysaccharides labeled with fluorophores can be used to detect the activities of endo-acting enzymes, enzymes that cleave a polymer mid-chain. These enzymes typically differ both in structure and in mode of hydrolysis from exo-acting enzymes that cleave a polysaccharide from the end of a chain [18]. Most investigations of enzyme activities in natural waters rely on measurements made with small substrate proxies (e.g. Ref. [24]), while studies of purified enzymes often use chemical techniques to detect the production of free reducing ends (e.g. Ref. [22]). Fluorescently labeled polysaccharides provide

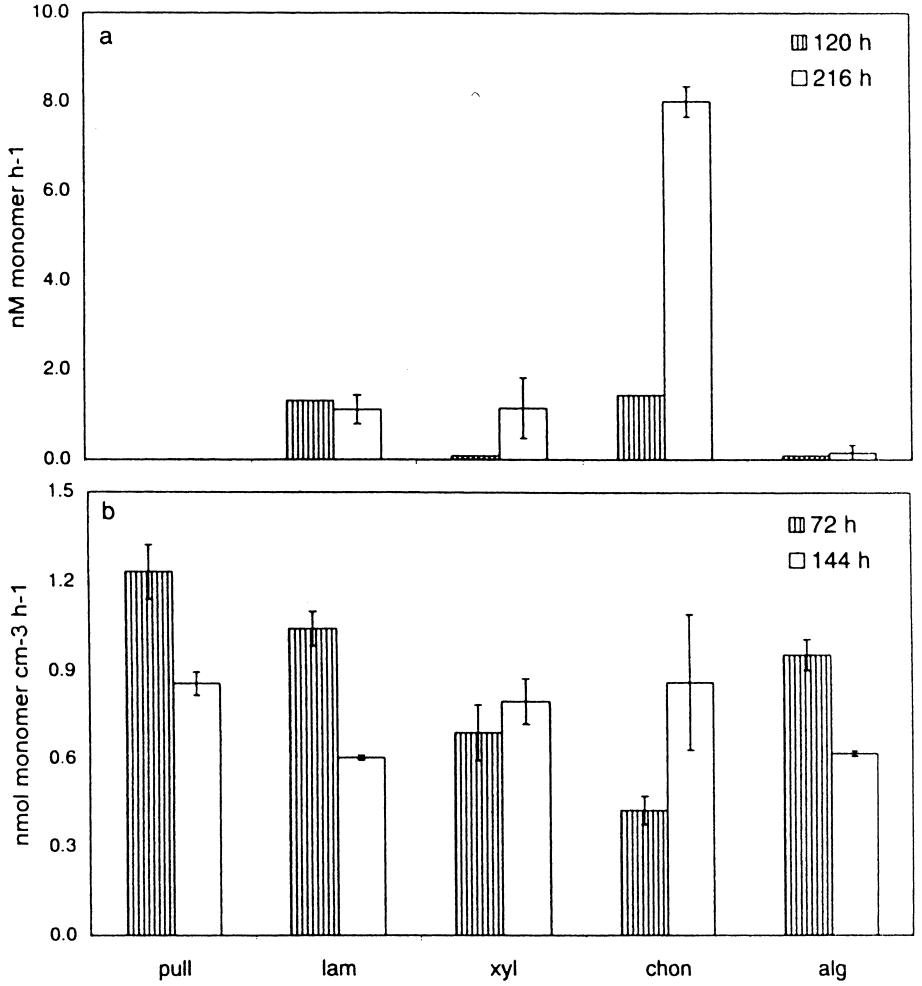

Fig. 3. Hydrolysis rates of five different fluorescently labeled polysaccharides in seawater (a) and homogenized sediments (b). Note that hydrolysis rates are plotted per liter seawater (a) and per milliliter sediments (b). Pull, pullulan; lam, laminarin; xyl, xylan; chon, chondroitin sulfate; alg, alginic acid.

an alternative means of investigating the activities of enzymes that hydrolyze a specific polysaccharide, without relying on proxies, and without the need for prior purification of enzymes.

When using fluorescently labeled polysaccharides for the detection of enzyme activities, however, a number of experimental factors must be considered. Measurement of enzyme activity using fluorescently labeled polysaccharides depends upon detection of changes in molecular mass of the polysaccharide as it is progressively hydrolyzed to smaller sizes. Because changes in the molecular mass distribution of the total pool of added substrate must be detected, starting with a substrate of uniform molecular mass is important. For this reason, the gel mesh of the column used and the fraction of polysaccharide collected during the fluorescent labeling procedure must be carefully considered. Ideally, the activated polysaccharide should elute in a tight band in the void volume of the column. For syntheses with xylan, laminarin, and fucoidan, G-25 gel (nominal exclusion limit for dextrans: 5000 M_r) was used, while G-50 gel (nominal exclusion limit for dextrans: 10 000 M_r) was used for pullulan, chondroitin sul-

fate, arabinogalactan, and alginic acid. For fucoidan in particular, when unlabeled polysaccharide was injected on a G-25 column, the polysaccharide eluted over a wide molecular mass range, spanning both the void volume and the included volume. For fucoidan, therefore, only the initial portion of the void volume of the column was collected. This fraction, once labeled, eluted in the void volume of the analytical chromatography system including G-75 gel (nominal exclusion limit for dextrans: 50 000). Dialysis in a membrane of appropriate molecular mass cut-off followed by lyophilization is an alternative method to obtain starting materials of more uniform molecular mass. This approach was used with alginic acid, and substrates prepared by extraction from natural sources such as algal cultures and commercial algal preparations, for example, generally also need to be dialyzed and lyophilized during the course of extraction and purification prior to labeling.

Detection limits for polysaccharide hydrolysis in complex media are affected by the nature of the medium, as well as by the quantity of substrate added to a given sample. A seawater sample obviously has fewer particles and a less complex 'background' than a sediment sample, simplifying detection of a signal. Substrate additions to sediments in this study were a factor of 50 higher than to seawater, yielding substrate concentrations that were readily detected in both types of samples. In part to compensate for lower substrate addition to the seawater samples, analyzed sample volume was typically a factor of 4–8 larger for seawater than for sediment samples. Given the typically extreme variability in composition and complexity of sediments from different locations, detection limits should be checked prior to initiating a set of experiments.

Substrate addition levels for detection of enzyme activity must in any case be balanced between exceeding a minimum detection limit, specific to the nature and characteristics of the sample, and avoiding excess substrate addition. Since enzyme activity is detected as a change in the molecular mass distribution of the total FLA-polysaccharide pool, adding excess substrate increases the incubation time required for hydrolysis to be detected. Furthermore, excess substrate addition and extended incubation times may permit growth of a subfraction of the microbial population, which can complicate interpretation of experimental data. The more rapid rate calculated for hydrolysis of xylan in seawater at 216 h compared to 120 h may be due to the fact that the changes in chromatographic behavior of the total pool of xylan were relatively small at the first time point, and substantial hydrolysis was detectable only after additional incubation (see [11,12] for further discussion of this point). Note, however that the significant increase in chondroitin sulfate hydrolase activity in seawater at 216 h compared to 120 h is likely due to induction of enzymes, as has been observed in a previous study [14].

It should be noted that enzymatic hydrolysis rates measured here are 'potential rates', since added substrate competes with naturally present substrate for enzyme active sites. Although total carbohydrate concentrations and concentrations of specific monosaccharides in acid-hydrolyzed samples can be determined in environmental samples, measurement of the naturally occurring concentration of a specific complex substrate is beyond current analytical capabilities. Typically, however, the concentration of added substrate is a high percentage of the total natural carbohydrate concentration in a sample. Hydrolysis rates measured with the fluorescently labeled substrates are therefore zero-order with respect to substrate concentration.

In the experiments described here, adding monomer-equivalent concentrations of the polysaccharides to a sample provided an independent means of comparing relative hydrolysis rates. To a first approximation, polymers of different initial sizes but equivalent monomer concentrations should be reduced to the monomer-size pool at the same rate if their respective extracellular enzymes also operate at the same rate. This comparison relies solely on chromatography, and is independent of the mathematical model used to calculate potential hydrolysis rate.

The patterns of extracellular enzyme activities detected in seawater and sediments differed substantially (Fig. 3). Although readily hydrolyzed in sediments, pullulan was not measurably hydrolyzed in seawater over the 216-h incubation, and alginic acid hydrolysis was barely detectable in seawater, even though activity in sediments was comparable to the other polysaccharides. Such differences in extracellular enzyme activities have previously been noted in a study carried out in the Skagerrak, the North Sea–Baltic Sea transition [14], although the present study

is the first to investigate alginic acid hydrolysis in seawater and sediments. Consistent with previous results, the differences in hydrolysis rates and patterns between seawater and sediments are not simply a function of total microbial numbers, which are typically two to three orders of magnitude higher on a volume basis in sediments than in seawater [25]. In Fig. 3, the units for seawater and sediments differ by a factor of 10^3, highlighting the differences in hydrolysis rates and patterns. As previously discussed [14], these differences may reflect fundamental compositional and functional differences between the microbial communities inhabiting these environments.

Investigation of such functional differences among complex microbial communities is still at very early stages. Approaches such as the use of fluorescently labeled polysaccharides provide insight into the activities of communities whose identities and metabolic capabilities are still largely unknown. A further major advantage of the general approach presented in this study is the relative ease of application in complex media and matrices. Since the dimensions, composition, and mobile phase for the gel filtration chromatography columns can be varied and the column materials are inexpensive, chromatographic conditions can be adjusted as needed. The need for sample manipulation and clean-up is minimized, since the system is generally robust, and when required, column replacement is straightforward and inexpensive. Furthermore, fluorescently-labeled polysaccharides may be applied in conjunction with a much wider range of spectroscopic techniques, including fluorescence polarization [20] and fluorescent resonance energy transfer (FRET), providing more detailed information on the dynamics of these macromolecules in solution.

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