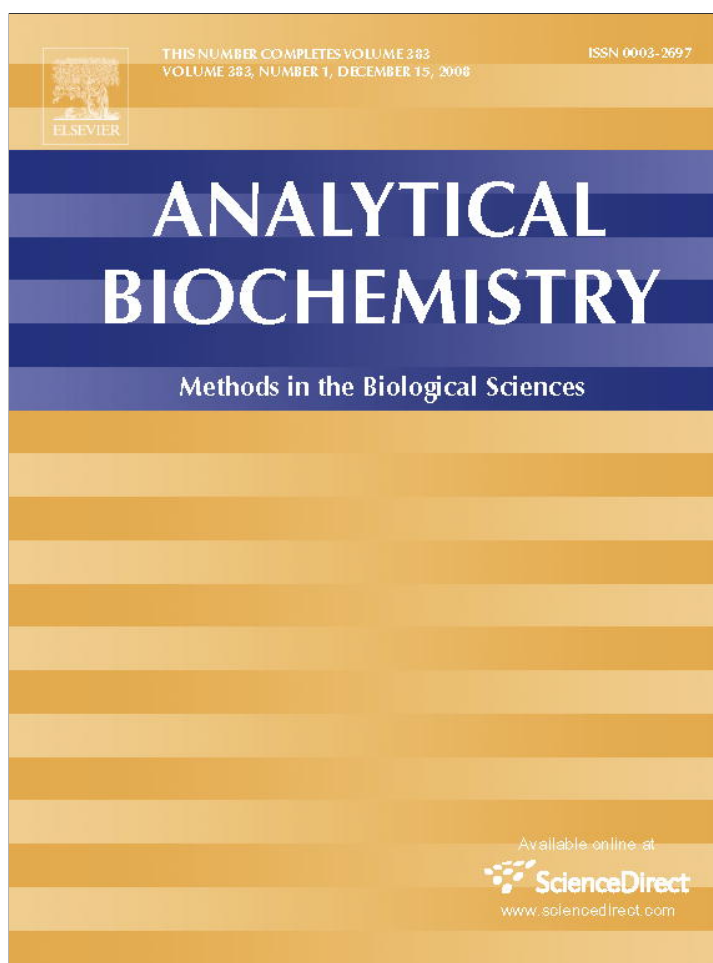


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Notes & Tips

Fluorescence anisotropy as a means to determine extracellular polysaccharide hydrolase activity in environmental samples

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ARTICLE INFO

Article history:

Received 4 August 2008

Available online 15 September 2008

ABSTRACT

Current approaches to measure the activities of microbial extracellular enzymes in aquatic environments are hampered by slow throughput or by differences between the structure of simple substrate proxies and macromolecules. Here we show that measurements of fluorescence anisotropy can be used to determine the hydrolysis rate of two fluorescently labeled polysaccharides, laminarin and xylan, in environmental samples. A simple analysis shows that the anisotropy of these fluorescently labeled polysaccharides can be approximated using a modification of the Perrin equation.

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The rates and structural specificities of microbial extracellular enzymes help to control the bioavailability and turnover of high-molecular-weight dissolved organic matter (DOM)¹ in natural waters [1]. The most widely used method to assess these enzyme activities is based on small substrate proxies (e.g., 4-methylumbelliferyl- β -D-glucopyranoside [2], 1-leucine-7-amido-4-methylcoumarin [3]) that consist of a monomer bound to a fluorophore that fluoresces when the fluorophore–monomer bond is cleaved. This method is inexpensive, precise, and rapid, but the structural differences between macromolecules and small substrate proxies mean that many aspects of enzyme structural selectivity cannot be explored, and the hydrolysis rates obtained with these substrate proxies may differ from those measured with the corresponding polymer [4]. Furthermore, small substrate proxies can diffuse into cellular periplasm, so they may record a combination of extracellular and periplasmic enzymatic activity [5]. An alternative approach is to measure directly, either by chromatographic analysis [6–8] or by electron paramagnetic resonance (EPR) spectroscopy [9], changes in the size of labeled macromolecules due to enzymatic hydrolysis. This approach has the advantage of using macromolecules rather than low-molecular-weight proxies and has been used to demonstrate that structurally similar polysaccharides may be hydrolyzed at very different rates in the same water sample [10–12]. However, chromatographic analysis for these approaches is relatively slow (30–90 min), and lack of portability of an EPR spectrometer precludes direct application in field studies. Here we demonstrate the utility of fluorescence anisotropy measurements as a rapid

(<2 min/sample) means to measure microbial extracellular enzyme activities through a comparison of the dependence of fluorescence anisotropy on molecular weight as determined by gel chromatography, permitting real-time data collection in the field. Analysis of the results using a simple model suggests that fluorescence anisotropy can be predicted with reasonable accuracy for a given molecular weight distribution in samples from fresh, estuarine, and marine environments.

Arnosti and coworkers [13] demonstrated that the anisotropy of fluorescently labeled (fl-) polysaccharides decreases as fl-polysaccharides are enzymatically hydrolyzed due to the decrease in anisotropy with decreasing molecular weight. This approach was used to track the hydrolysis of two fl-polysaccharides, fl-laminarin and fl-xylan, incubated in pore waters from sediments of the Chesapeake Bay. However, this previous study did not quantitatively compare anisotropy changes with chromatographic analysis of hydrolysis.

Laminarin (6 kDa) and xylan (8 kDa) were obtained from Fluka and labeled with fluorescein amine by the method of Glabe and coworkers [14] as modified by Arnosti [7]. The labeling density (number of fluorophores per monomer) for laminarin and xylan is typically approximately 0.01, meaning that individual molecules with multiple labels are very rare [7]. (Other polysaccharides are larger and/or receive label more readily, so that individual polysaccharide molecules may typically receive multiple fluorophores.)

Surface water samples were collected from fresh water at the head of the Chesapeake Bay (surrounded by Maryland and Virginia on the U.S. east coast), two estuarine sites in the Chesapeake Bay, and a coastal ocean site, as described in Ref. [12]. Fl-polysaccharides were added to triplicate, unfiltered water samples to a concentration of 3.5 $\mu\text{mol L}^{-1}$ (monomer equivalent) in samples

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¹ Abbreviations used: DOM, dissolved organic matter; EPR, electron paramagnetic resonance; fl-, fluorescently labeled; GPC, gel permeation chromatography.

intended for gel permeation chromatography (GPC) analysis, and $12.4 \mu\text{mol L}^{-1}$ (fl-laminarin) or $2.4 \mu\text{mol L}^{-1}$ (fl-xylan) in samples for fluorescence anisotropy analysis. The samples were incubated at 22°C for 10 days. Subsamples were periodically withdrawn, $0.2 \mu\text{m}$ filtered, and immediately frozen.

The extent of hydrolysis, equal to the number of free reducing ends produced via hydrolysis, was calculated from GPC chromatograms as in Arnosti [6]. (The GPC data and conditions are reported in Ref. [12].) To calculate extent of hydrolysis, each chromatogram was divided into five molecular weight “bins” spanning the range from 150 kDa to free fluorophore. The amount of carbohydrate in each size bin was assumed to be proportional to the fluorescence signal integrated across that bin. The extent of hydrolysis was calculated as the minimum number of enzymatic “cuts” required to produce the observed molecular weight distribution at each time point, starting from the distribution observed at the initial time point.

Fluorescence anisotropy measurements were made using an ISS K2 frequency domain spectrofluorometer in steady-state counting mode, as described in Arnosti and coworkers [13], with excitation and emission set to 498 and 530 nm, respectively, and a measurement time of 100 s. Fluorescence lifetime measurements were acquired with the same instrument employing procedures described previously [15].

Fluorescence anisotropy of each substrate was well correlated to the extent of hydrolysis determined by GPC (laminarin $r^2 = 0.951$, xylan $r^2 = 0.909$) (Figs. 1A and 1B). One fl-laminarin sample was excluded from this comparison due to high variability among replicates in the GPC measurement. Representative time courses of GPC chromatograms from the coastal ocean sampling station showing the progression from unhydrolyzed polysaccharide to monomeric (or near-monomeric) form are shown in Figs. 1C and 1D.

The roughly linear decrease of anisotropy with respect to the GPC-measured extent of hydrolysis and anisotropy is somewhat

surprising given that the observed anisotropy arises from an ensemble of polysaccharides with a range of sizes. The relationship between anisotropy and molecular weight for a single polysaccharide is nonlinear (see below), and the relationship between the size of a polysaccharide and the number of enzymatic cuts needed to arrive at that molecular weight is also nonlinear, so this result was not expected. Nevertheless, this linear relationship held across the entire range of molecular weights for the polysaccharides studied here.

To examine this relationship in more detail, the following analysis was employed. Fluorescence anisotropy r of a rigid sphere is given by the Perrin equation [16],

$$R = \frac{r_0}{1 + \frac{\tau}{\theta}}, \quad (1)$$

where r_0 is the fundamental anisotropy (0.375 for fluorescein [17]), τ is fluorescence lifetime, and θ is rotational correlation time. θ is given by

$$\theta = \frac{\eta M(\bar{v} + h)}{RT}, \quad (2)$$

where η is solvent viscosity, M is molecular weight, \bar{v} is specific volume of the solute, h is hydration, R is gas constant, and T is temperature. Fluorescence lifetime data for the intact labeled polysaccharides, collected at pH 8.4 in 50 mM borate buffer, were well fit by a biexponential decay; for xylan, fluorescence lifetimes were 3.4 ns (53.7% of steady-state intensity) and 0.76 ns (46.3%), whereas for laminarin, fluorescence lifetimes were 3.8 ns (86.3%) and 0.94 ns (13.7%). The dual lifetimes suggest two distinct populations, likely arising from fluorophores in differing environments within the polysaccharide. The specific volume of a range of neutral carbohydrates is close to 0.649 ml g^{-1} [18], and assuming that half of hydroxyl groups in the polysaccharide are bound to a water molecule [19], we estimate h to be 0.22 for laminarin and 0.20 for xylan.

The observed anisotropy for a mixture of fluorophores is given by

$$r = \sum_i f_i r_i, \quad (3)$$

where f_i represents the fraction of steady-state fluorescence intensity having anisotropy r_i . Anisotropy as a function of molecular weight, therefore, was modeled as

$$R = \sum_i \frac{f_i r_0}{1 + \frac{\tau_i}{\theta}}, \quad (4)$$

with f_i as the steady-state intensity for each observed lifetime, $\eta = 0.94 \text{ cP}$, $\bar{v} = 0.649 \text{ ml g}^{-1}$, $h = 0.42 \text{ ml g}^{-1}$, and $T = 300 \text{ K}$. For the purpose of this analysis, we assume that fluorescence lifetimes are invariant with respect to molecular weight, although a more complete treatment could include potential changes in fluorescence lifetime as a function of polysaccharide molecular weight.

For an ensemble of polysaccharides of varying molecular weights M ,

$$r = \sum_M f_M \sum_i \frac{f_i r_0}{1 + \frac{\tau_i}{\theta_M}}, \quad (5)$$

where f_M is the mass fraction of the ensemble having molecular weight M . f_M for each chromatogram was determined by assigning a molecular weight to each elution time (the fluorimeter returned measurements at a frequency of 5 Hz) based on a fit of elution time to the logarithm of the molecular weight of fl-dextran size standards, with a minimum possible molecular weight set to 180 Da.

The anisotropies calculated according to Eq. (5) are well correlated with observed anisotropies: laminarin $r_{\text{observed}} = 1.01 \pm 0.18 \times r_{\text{calculated}} - 0.244 \pm 0.064$, $r^2 = 0.925$; xylan $r_{\text{observed}} = 1.16 \pm$

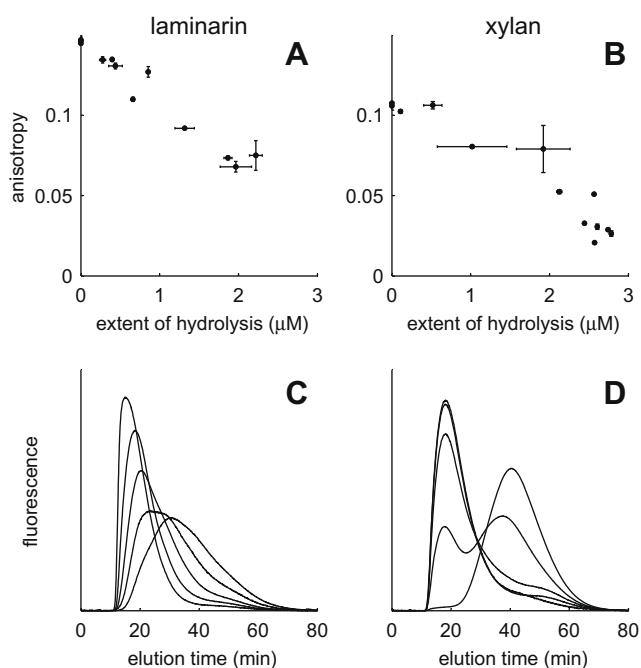


Fig. 1. Anisotropy versus extent of hydrolysis for partially hydrolyzed fl-laminarin (A) and xylan (B) throughout the time course of the experiment. Error bars are ± 1 standard deviation for replicate samples. Also shown are representative chromatograms from each time point at the marine station for fl-laminarin (C) and fl-xylan (D).

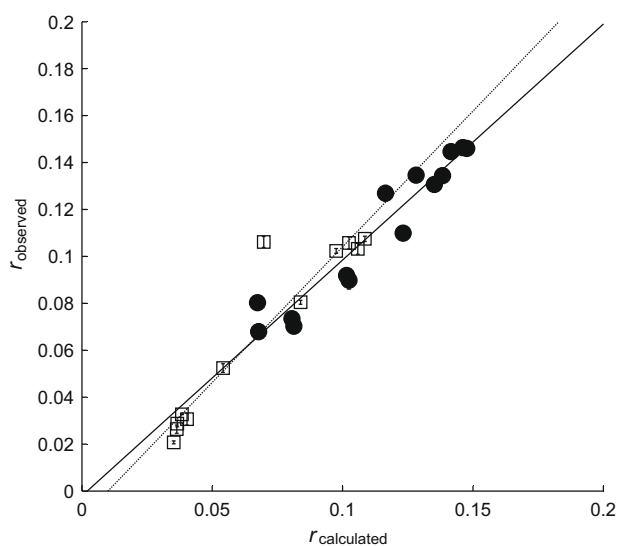


Fig. 2. Measured anisotropy versus calculated anisotropy for fl-laminarin (filled circles) and fl-xylan (unfilled squares). Error bars are ± 1 standard deviation for replicate samples and are generally smaller than the data points.

$0.28 \times r_{\text{calculated}} - 0.300$, $r^2 = 0.895$ (reported error is 95% confidence interval). The slope of each of these relationships is close to 1, but in both cases observed anisotropy was considerably lower than calculated anisotropy. Fl-macromolecules almost always show lower anisotropy than would be calculated using a rigid sphere approximation because internal flexibility of the macromolecule and motion of the fluorophore relative to the macromolecule cause faster rotational diffusion than would be predicted for a rigid sphere [20].

The results of Eq. (5) were corrected for the offset between calculated and observed anisotropy of the unhydrolyzed polysaccharide:

$$r = r_{\text{calculated}} - (r_{\text{calculated,unhydrolyzed}} - r_{\text{observed,unhydrolyzed}}) \quad (6)$$

This correction yields a fit line that passes approximately through the origin (laminarin intercept = -0.003 ± 0.021 , xylan intercept = -0.024 ± 0.021) (Fig. 2). The fact that the offset between calculated and measured anisotropy is fairly constant with respect to molecular weight is consistent with a model in which depolarization arises mainly from two sources: rotation of the polysaccharide as a whole, approximating rigid body rotation, and rotation of the fluorophore independent of the polysaccharide (e.g., rotation about the fluorophore–saccharide bond).

The success of this model indicates that Eq. (6) reasonably approximates the anisotropy of a disperse ensemble of fluorescently labeled polysaccharides, oligosaccharides, and monomers in freshwater, estuarine, and marine environments. A complete theoretical description of the anisotropy of such an ensemble would need to account for a great many variables, including pH and ionic strength of the medium, internal flexibility of polysaccharides, potential changes in fluorescence lifetime as a function of molecular weight, and the change in axial ratio of the polysaccharides as a function of molecular weight. The success of the simple model here indicates that those complicating factors may be ignored in planning experiments that use fluorescence anisotropy to measure hydrolysis rates of labeled macromolecules. The speed and ease of measuring fluorescence anisotropy, combined with the fact that a spectrofluorometer may be paired with a multiwell plate reader and carried to a field station or used aboard a research

vessel, suggests that fluorescence anisotropy measurements may open the door to experiments probing the controls on enzyme activities in the environment that involve large numbers of samples and real-time data analysis.

Acknowledgments

The authors thank Thomas Boyd and the captain and crew of the RV *Cape Henlopen*. A.D.S. received funding from the U.S. Environmental Protection Agency (EPA) under the Science to Achieve Results (STAR) Graduate Fellowship Program. The EPA has not officially endorsed this publication, and the views expressed herein might not reflect the views of the EPA. Funding was provided to A.D.S. and C.A. by the National Science Foundation (NSF, OCE-0323975) and a grant from the Petroleum Research Fund. P.G. and N.V.B. were supported in part by the Office of Naval Research (ONR) and the NSF. Ship time was funded by the Naval Research Laboratory (NRL)/ONR platform support program.

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