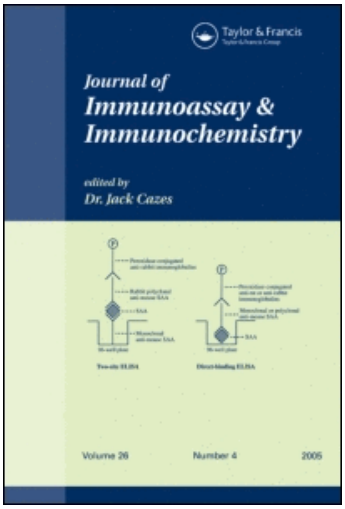

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Development of an Indirect Competitive Enzyme-Linked Immunosorbent Assay to Detect Extracellular Polymeric Substances (EPS) Secreted by the Marine Stromatolite-Forming Cyanobacteria, *Schizothrix sp.*

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ABSTRACT

An indirect competitive enzyme-linked immunosorbent assay was developed using polyclonal antibody to detect extracellular

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polymeric secretions (EPS) produced by the marine stromatolite-forming cyanobacteria, *Schizothrix sp*. The cross-reactivity of this assay with other EPSs and polymers were low (<0.5%). This assay can detect *Schizothrix sp*. EPS as low as 0.5 ng/mL. Intra-assay and inter-assay comparisons showed that coefficient variations were low, ranging from 3.34 to 10.30% and from 6.30 to 12.8%, respectively, for standards between 2 and 1000 ng/mL. Also, the seawater matrix effect was negligible. Our results indicated that this assay is a useful tool for quantification of *Schizothrix sp*. EPS in a range of ng/mL.

INTRODUCTION

Sediment biofilms typically contains abundant extracellular polymeric secretions (EPS) which are the primary structuring agent for microbial microenvironments and control the physical properties of biofilms.[1] Dissolution of EPS components of biofilms often contribute to the dissolved organic carbon (DOC) in the overlying water-column. This may be in the form of dissolved or colloidal organic matter.[2]

Modern subtidal and intertidal marine stromatolites are found in the Exuma Cays, Bahamas.[3] The cyanobacterium, *Schizothrix sp*., is a dominant species that secretes abundant EPS in this system which play important roles in binding sand grains and $CaCO_3$ precipitation.[4–8] EPS contain, predominantly, acidic polysaccharides and proteins.[9] However, it is not well known how much EPS contribute to the DOC pool in the surrounding environment of marine stromatolites. Therefore, in order to understand the contribution of this EPS to the DOC cycle in the Exuma Cays, Bahamas, it is important to develop a specific probe for the quantification of *Schizothrix sp*. EPS, hereafter referred to as "SEPS."

The binding specificity of lectins such as Concanavalin A (ConA) and wheat germ agglutinin (WGA) has been used previously to quantify EPS in biofilms.[10–12] Although those lectins recognize the specific sequence of polysaccharides common among EPS,[13] it is not possible to distinguish species specific EPS.

In this study, we report the development of *Schizothrix sp*. EPS-specific polyclonal antibody and the indirect competitive enzyme linked immunosorbent assay (ELISA) for the quantification of *Schizothrix sp*. EPS within the low ng/mL concentration range.

EXPERIMENTAL

Schizothrix sp. Culture and EPS Extraction

The cyanobacteria, *Schizothrix sp.*, was isolated from marine stromatolites at Highborne Cay, Bahamas. The species was grown in a CHU-10 medium[14] consisting of 0.004 M $Na_2SiO_3 \cdot 9H_2O$, 0.006 M $Ca(NO_3)_2 \cdot 4H_2O$, 0.014 M K_2HPO_4, 0.025 M $MgSO_4 \cdot 7H_2O$, 0.05 M Na_2CO_3, 0.012 M Fe-EDTA, 3.7×10^{-8} M B_{12}, 4×10^{-7} M biotin, and 5.9×10^{-7} M thiamine in seawater of 32 ppt salinity on a light:dark cycle of 12:12 h, at approximately 100 μEinsteins. Bahamian sediment, consisting of well-sorted $CaCO_3$ ooids (mean grain size 100–250 μm), was collected from the study site, sterilized, cleaned in sodium hypochlorite, and rinsed thoroughly in distilled H_2O. The sediment was added to culture flasks to a depth of approximately 0.5 cm as a substratum for growth. Cultures were grown for several weeks until a firm mat of cyanobacteria was present on the surface of the sediment and the culture medium was discarded. Then, the culture was suspended, stirred, and heated to 40°C for at least 30 min to strip EPS from the cyanobacteria. The suspensions were centrifuged at 12,000 rpm for 15 min in order to shear remaining EPS from cells. To remove small-molecular weight (MW) components, the supernatant was dialyzed (MW cutoff 14,000) in de-ionized water, with constant stirring, for 48 h. The dialyzed solution was then lyophilized and stored at −70°C. To test the specificity of antibody to *Schizothrix sp.* EPS, other cyanobacteria isolated from the same marine stromatolites samples (red *Phormidium sp.*, *Plectonema sp.*, *Synechocystis sp.*, *Oscillatoria sp.*, and *Solentia sp.*) were cultured and their EPS were extracted by the same method described above. SEPS were fractionated into two different size fractions (>100 kD and <100 kD) using Microcon YM-100 centrifugal filter units (Millipore, Bedford, MA, USA).

Antibody Production

Polyclonal antibodies were raised in a New Zealand White male rabbit against laboratory-cultured *Schizothrix sp.* EPS. Rabbits were initially injected with 100 μg of the antigen suspended in Freund's complete adjuvant. Over a four-month period, three additional injections were performed in similar fashion using Freund's incomplete adjuvant. Two weeks after the third injection, the rabbit was killed under

anesthesia and sera were separated from blood. The IgG fraction was purified by using a HiTrap Protein A HP column (bed volume: 1 mL, Amersham Pharmacia, Uppsala, Sweden) and stored at −70°C until use.

Indirect Competitive Enzyme-Linked Immunosorbent (ELISA) Assay for *Schizothrix sp.* EPS

Selection of ELISA Conditions

The coat concentration that gave maximum inhibition with 20 μg/mL of SEPS was chosen as the optimal coat concentration (Fig. 1). Bovine serum albumin, 0.1%, in Tris buffer saline (BSA-TBS) as a blocking solution, gave the best standard curve among other concentrations of BSA-TBS (0.5% and 1%) and other blocking solutions, such as SuperBlock Blocking buffer in TBS (Pierce, Rockford, IL, USA). Anti serum concentration, selected at 1:1000, gave a desired absorbance of around 1.0 and was found to be low enough to become the limiting factor in the assay giving an easily measurable response.

ELISA Procedure

All incubations were carried out at ambient temperature (22–25°C). SEPS standards were prepared by diluting a stock solution of SEPS

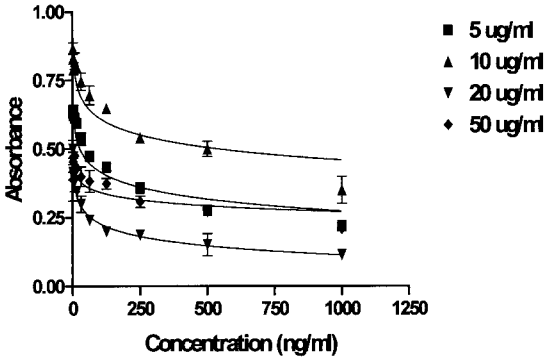

Figure 1. Comparison for *Schizothrix sp.* EPS coating concentration of the indirect competitive ELISA.

1 mg/mL in TBS with 0.1% BSA-TBS to a final concentration of 1–1000 ng/mL.

1. Microplates were coated overnight on the shaker with 100 μL/well of 10 μg/mL solution of coating antigen, SEPS, in 50 mM carbonate buffer (pH 9.6).
2. Plates were emptied and blocked with 300 μL/well of 0.1% BSA-TBS for 1 h to eliminate nonspecific binding by blocking the plastic surface where EPS was not bound.
3. After 1 h incubation, plates were emptied and washed three times with TBS containing 0.05% Tween-20 in TBS (TTBS). 75 μL/well of 1:1000 anti-SEPS IgG and 75 μL/well of varying concentrations of EPS (0–1000 ng) were added and incubated overnight on the shaker.
4. Next day, the solution was removed and plates were washed with TTBS three times. One hundred and fifty microliters per well of goat anti-rabbit IgG-alkaline phosphatase in 0.1% BSA-TBS was added and incubated for 1 h at room temperature. Then, the solution was removed and plates were washed four times with TTBS.
5. Substrate, *p*-nitrophenylphosphate solution was added, followed by the addition of stopping solution (0.5 M NaOH) after 30 min of incubation on the shaker. The absorbance at 405 nm was then read with a Biotek Powerwave 200 microplate reader and concentration of SEPS was calculated automatically using software on the basis of the concentrations of a set of standards included on each plate.

Determination of Cross-Reactivities

The ability of antiserum to recognize several related polymers was tested by performing competitive assays and determining their respective IC_{50} values (50% inhibition of control). Cross-reactivity was calculated as (IC_{50} of *Schizothrix sp.* EPS/IC_{50} of related polymer) × 100.

The related polymers used in this study were: EPS isolated from five cyanobacterial species (red *Phormidium sp.*, *Plectonema sp.*, *Synechosystis sp.*, *Osillatoria sp.*, and *Solentia sp.*), Dextran (74 kD), Dextran (167 kD), Gum Xanthan, Carrageenan Type I, Carrageenan Type II, Curdlan (β-[1→3]-D-Glucan), *Schizothrix sp.* EPS fraction (>100 kD), and *Schizothrix sp.* EPS fraction (<100 kD).

Analysis of Water Samples

Water samples were spiked with SEPS to evaluate the potential matrix effect on the ELISA. The water samples were 0.45 μm membrane filtered artifical seawater (ASW). For ELISA analysis, 2 mL of ASW was spiked with known concentrations of SEPS covering the quantitative working range (2 ng/mL, 4 ng/mL, 10 ng/mL, and 20 ng/mL). Each determination was run in quadruplicate, and the mean absorbance was interpolated in a standard curve performed in the same ELISA plate. Data represent the mean of four independent determinations.

RESULTS AND DISCUSSION

In the indirect competitive ELISA protocol, our research showed that, in order to be in a portion of the response curves, a coating antigen concentration of 20 μg/mL and antiserum dilution of 1:1000 were needed. The typical standard curve is seen in Fig. 2.

The reproducibility of the method, based on a set of SEPS standards run on different plates on the same day (intra-assay), and on different days (inter-assay), is summarized in Table 1. The intra-assay coefficient of variation (CV) of the standards used for calibration of the assay ranged from 3.34 to 10.30% for standards between 2 and 1000 ng/mL. The inter-assay CV ranged from 6.30 to 12.8% for standards between 2 and 1000 ng/mL. At the lowest standard concentration of 1 ng/mL, the CV

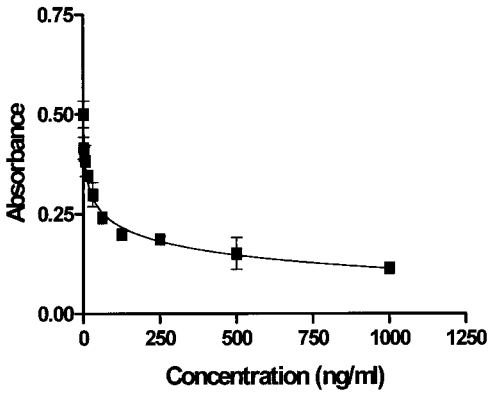

Figure 2. Typical standard curve of the indirect competitive ELISA for *Schizothrix sp*. EPS.

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Table 1. Intra-assay and inter-assay variation of *Schizothrix sp.* EPS (SEPS) standards.

SEPS (ng/mL)	Intra-assay ($N=4$) % inhibition (mean)	Variation		Inter-assay ($N=3$) % inhibition (mean)	Variation	
		SD	CV (%)		SD	CV (%)
1	24.9	0.028	25.0	25.6	0.117	25.5
2	25.6	0.045	7.30	35.7	0.087	6.30
15.7	34.6	0.059	10.30	54.3	0.102	12.8
62.5	53.8	0.016	3.34	63.4	0.130	11.8
125	76.8	0.035	8.22	78.7	0.120	9.9
250	81.3	0.022	6.51	80.9	0.136	8.9
500	86.5	0.015	5.64	87.6	0.124	7.8
1000	89.1	0.020	9.48	88.9	0.089	6.8

of both intra-assay and inter-assay increased to 25.0 and 25.5%, respectively. Due to high variability with the low (1 ng/mL) standard, the sensitivity of the assay was set at 2 ng/mL. The detection limit was at least 0.5 ng/mL below these conditions.

Antibody specificity was determined by the indirect competitive ELISA, in which *Schizothrix sp*. EPS competitors were presented in the assay to compete with the binding of SEPS. EPS from different cyanobacterial species and polymers, that were used to test the cross-reactivity, showed no more than a 0.5% cross-reactivity. Although red *Phormidium sp*. EPS has a similar molecular weight to SEPS, it had a low cross-reactivity (0.2%) with anti-SEPS antibody in this assay. Also, two different size fractions from SEPS (>100 kD and <100 kD) had low cross-reactivities (0.3 and 0.5%, respectively) (Table 2). Thus, this antibody recognizes a specific structural feature in the SEPS. To our knowledge, there has been little research in regard to quantifying species-specific EPS using antibodies in marine environments because of its poor immunogenicity. Nanninga et al.[15] reported that EPS from the unicellular alga, *Emiliania huxleyi*, was quantified by ELISA, using polyclonal antibody, as low as 20 ng/mL. Although antibodies have a high specificity for isolated EPS, the immunogenicity of EPS is rather poor compared to that of proteins or lipopolysaccharides; a factor that results in a low antibody yield.[14] However, Kawaguchi and Decho[9,17] successfully produced a polyclonal antibody for SEPS and applied it to localize SEPS in nanoplast sections of marine stromatolite using confocal laser scanning

Table 2. Cross-reactivities of various EPS and polymers.

Polymers	Cross-reactivity (%)
Red *Phormidium sp*.	0.2
Plectonema sp.	0.3
Synechosystis sp.	0.1
Osillatoria sp.	0.1
Solentia sp.	0.1
Dextran (74 kD)	0.2
Dextran (167 kD)	0.2
Gum Xanthan	0.3
Carrageenan Type I	0.1
Carrageenan Type II	0.1
Curdlan (β-[1→3]-D-glucan)	0.1
Schizothrix sp. EPS (>100 kD)	0.3
Schizothrix sp. EPS (<100 kD)	0.5

Table 3. Recovery of SEPS from spiked water samples.

Sample	SEPS added (ng/mL)	SEPS recovered (ng/mL)	SD[a] (ng/mL)	CV[b] (%)	Recovery (%)
Sea water	2	2.0	0.063	16.8	100.00
	4	4.2	0.126	14.7	104.00
	10	10.3	0.570	13.5	101.5
	20	20.3	0.543	8.7	102.5

[a]Standard deviation.
[b]Intra-assay coefficient of variation.

microscopy. In this study, we successfully developed the indirect competitive ELISA to quantify SEPS using polyclonal antibody.

It is generally known that immunoassays can be used to rapidly quantitate various compounds. However, the antigen–antibody interaction may be affected by a variety of compounds.[18] Since SEPS may be dissolved or suspended dissolved, or suspended in seawater under natural condition, we evaluated the method by spiking seawater samples with several amounts of SEPS for the performance of the assay. Some seawater samples were spiked at several concentrations of SEPS, covering the optimized working range (2, 4, 10, and 20 ng/mL). Determinations were made in quadruplicate and the mean absorbance was used to estimate SEPS concentration by interpolation in the TBS standard curve performed in the same plate. Results of analytical data, expressed as the percentage of recovery, are summarized in Table 3. Very similar CV's were found, ranging from 8.7 to 16.8%. The ELISA data showed a slight tendency for overestimation, compared to theoretical concentration levels.

In conclusion, we developed the indirect competitive ELISA for quantification of EPS from stromatolite-forming cyanobacteria, *Schizothrix sp*. Our results demonstrate that the indirect competitive ELISA is a useful tool for quantification of species-specific EPS, to levels as low as 0.5 ng/mL.

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