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Detection of Iron(III)-Binding Ligands Originating from Marine Phytoplankton Using Cathodic Stripping Voltammetry

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The sample preparation and analytical methodology are described for detecting biologically produced iron(III)-binding ligands in laboratory cultures of coastal marine phytoplankton. The iron(III)-binding ligands from the culture media were purified by passage through a column packing with a hydrophobic absorbent. The concentrations and stability constants of the ligands were determined by adsorptive cathodic stripping voltammetry with competitive ligand equilibration. The analytical results of the cultivated cultures suggest that eukaryotic phytoplankton would produce iron(III)-binding ligands in analogy with other microorganisms.

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Introduction

It is generally accepted that dissolved iron in the oceans is overwhelmingly bound to strong organic ligands.[1-5] Although strong organic ligands have been poorly characterized, they can be potentially produced biologically as metabolites from metabolic pathways or as byproducts of lysis processes. Much attention has been paid to bacterially produced siderophores, low molecular weight ligands with high affinity for iron,[6-8] as the major constituent of iron(III)-binding ligands in seawater.[9] Several researchers have revealed that prokaryotic species, such as cyanobacteria and heterotrophic bacteria, release siderophores and facilitate iron uptake.[10-14] On the other hand, certain eukaryotic phytoplankton also release iron(III)-binding ligands, of which the biological role is unknown.[15-17,31]

There are several conventional methods for the determination of biogenic iron(III)-binding ligands in bacterial cultures.[18] Various reactions, such as the CAS assay,[19] the Csaky test,[20,21] periodate oxidation[22] and the Arnows reaction,[23] were used previously. The detection limits of these methods depend on absorbance measurements by a spectrophotometer. However, recent studies have suggested that the release of ligands from phytoplankton is lower than that of other microorganisms.[16,17] The colorimetric methods may not be sufficiently sensitive to measure iron(III)-binding ligands from phytoplankton.

On the other hand, a wide range of analytical methods is proposed for speciation studies in natural waters.[24,25] In particular, electrochemical techniques have been developed for measurements of organically complexed iron(III) in seawater during the last decade.[1-3] These techniques are based on competitive equilibration between natural iron(III)-binding ligands and a well-characterized added ligand, followed by adsorptive cathodic stripping voltammetry. Titration of the ligands with iron(III) provides information about the concentrations and the stability constants for iron(III) at low concentration levels of 10^{-7} to 10^{-11} M.

In this paper we report on an analytical method for iron(III)-binding ligands in laboratory cultures of coastal marine phytoplankton. The ligands were separated by solid-phase extraction with a Sep-Pak cartridge column, and determined by adsorptive cathodic stripping voltammetry with competitive ligand equilibration (CLE/ACSV). Some results of coastal eukaryotic phytoplankton cultures are described, which suggest that extracted compounds from the culture media showed iron(III)-binding affinities by electrochemical analysis.

Experimental

Instrumentation and reagents

Voltammetric measurements were made using a voltammetric analyzer (PAR263A, EG&G, USA) with a mercury drop electrode (PAR303A, EG&G). The working electrode was a large mercury drop, the reference electrode was Ag/AgCl in 3 M KCl saturated with AgCl, and the counter electrode was a platinum wire. For a measurement of ACSV, a stock solution of 1 mM 1-nitroso-2-naphthol (NN; Wako, Osaka) was prepared in methanol. A stock solution of iron(III) was prepared by dissolving $FeCl_3 \cdot 6H_2O$ (Nacalai Tesque, Kyoto) in 1 M HCl (TAMAPURE-AA-10, Tama Chemicals, Tokyo) and standardized by using inductively coupled plasma atomic emission spectrometry (Optima 3000, Perkin-Elmer, USA). Stock solutions of chelating ligands were prepared by dissolving the corresponding compounds (ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA) and diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), Dojindo Molecular Technologies) in 0.1 M sodium hydroxide. They were diluted to the desired concentrations. Artificial seawater was prepared according to Fleming,[26] and deionized water using an E-pure system (Barnstead) was used throughout. Other reagents were of analytical reagent grade or better.

Column selection for separation of iron(III)-binding ligands

Thirteen Sep-Pak cartridge columns (Sep-Pak plus, Waters)

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were tested, of which the absorbents are divided into three types: non-ionic hydrophobic (C18, tC18, C8, PS-2 and CN), hydrophilic (Diol, Silica, Florisil and Alumina N), and ion exchange (Alumina A, Alumina B, NH_2 and Accell QMA). These columns were pretreated according to the manufacturer specifications, and rinsed with 20 mL of artificial seawater. Two model ligand solutions were prepared by dissolving natural iron(III)-binding ligands, desferrioxamine B (Sigma) and rhodotorulic acid (Sigma), to deionized water to give 0.30 μM. Two hundred milliliters of the model ligand solutions were passed through each column, respectively. The extracted compounds were eluted with 20 ml of the prescribed eluting solution, and determined by the CAS assay described below.

Culture experiments and sample treatments

Axenic cultures of *Chattonella antiqua* NIES-1, *Heterosigma akashiwo* 893 (Raphidophyceae), *Skeletonema costatum* NIES-16 (Bacillariophyceae) and *Rhodomonas ovalis* (Cryptophyceae) were used. These species are classified as coastal red-tide species. All procedures for culture experiments were performed under a clean bench (class 100), and culture media were sterilized by autoclaving (121˚C, 30 min). Experimental cultures were grown at 20˚C under a 12:12 h L/D photoperiod at a light intensity of 180 μmol photon m^{-2} s^{-1} provided by cool white fluorescent lights.

Prior to the experiments, the algal cultures were maintained in modified f/2 media, reducing the concentrations of iron (Fe(III), 200 nM) and omitting the chelating ligands (Table 1). They were grown to the exponential phase, respectively. Six hundred milliliters of the modified f/2 medium in 1-L capacity acid-washed polycarbonate bottles were equilibrated at 20˚C for 48 h, and inoculated with the acclimated exponential phase cells of phytoplankton, which resulted in 20 cells mL^{-1}. The cultures were grown until the population reached the end of the exponential phase, usually 1 – 3 weeks. Phytoplankton growth was followed by measuring spectrophotometrically on a UV-VIS spectrophotometer at 540 nm, and correlated with an established cell density-to-absorbance ratio to estimate the cell number. The cell number was counted directly by using a microscope. The axenic nature was verified by DAPI direct straining and an examination under an epifluorescent microscope.[27]

For collecting iron(III)-binding ligands, cells of phytoplankton were removed by centrifugation at 3000-times gravity for 10 min. Two hundred milliliters of the resulting supernatants were filtered through 0.22-μm polycarbonate filters. They were passed through a preconditioned Sep-Pak C18 cartridge column at 10 ml/min. This extraction procedure was repeated three times. After washing with 100 ml of deionized water, the extracted compounds were eluted with 40 ml of methanol, respectively. The collected eluents from the three columns were evaporated to dryness by rotary evaporation (< 40˚C, < 30 mmHg). The residue was redissolved in 20 ml of a 0.01 M NaOH solution and stored at 4.0˚C in a refrigerator until analysis.

Measurement of iron(III)-binding ligands

Concentrations of iron(III)-binding ligands were titrated with iron while monitoring of the labile iron concentrations. The term “labile iron” is defined in this paper as the iron which reacts with the added NN within 5 h. To measure the labile iron, we revised a CLE/ACSV method based on previous studies.[1,2,28,29] Under a class-100 clean condition, 9.5 ml aliquots of a 3.5% NaCl solution were pipetted into FEP-Teflon voltammetric cell cups, and premixed with 0.5 ml of samples containing iron(III)-binding ligands, 154 μl of 0.65 M HEPES buffer (pH 8) and 100 μl of a 1 mM NN stock solution. Iron(III) stock solutions were added to the cups, yielding concentrations from 0 to 40 nM, and the added iron was allowed to equilibrate with the iron(III)-binding ligands for 5 h at room temperature. After the samples were deaerated for 4 min by purging with 0.1 μm-filtered N_2 gas, deposition was carried out for 5 min at –0.15 V with stirring. The potential was scanned using the differential pulse mode from –0.05 to –0.9 V at 10 mV/s. The labile iron concentrations were determined from the height of the reduction peak of iron above the baseline at –0.55 V. The stability constant for complexation of iron(III) by NN was calibrated for a 3.15% NaCl solution by ligand competition against EDTA and DPTA, as described by van den Berg (1995).[2]

Table 1 Compositions of a modified f/2 medium using artificial seawater

$NaNO_3$	8.8 μM	$CoSO_4 \cdot 7H_2O$	4.3 pM
$NaH_2PO_4 \cdot 2H_2O$	0.43 μM	$ZnSO_4 \cdot 7H_2O$	3.5 pM
$Na_2SiO_3 \cdot 9H_2O$	0.35 μM	$MnCl_2 \cdot 4H_2O$	91 pM
Thiamine HCl	0.30 nM	$CuSO_4 \cdot 5H_2O$	2.8 pM
Biotin	21 pM	$Na_2MoO_4 \cdot 2H_2O$	2.9 pM
Vitamin B_{12}	3.7 pM	H_2SeO_3	100 nM
HEPES	5.0 mM	$FeCl_3 \cdot 6H_2O$	0 – 200 nM
pH 8		EDTA analogues	0 – 20 nM

The concentrations of the total dissolved iron were determined directly by graphite furnace atomic absorption spectrometry (Z-8100, Hitachi), and by ACSV after UV digestion for 2 h using a 400 W high-pressure mercury vapor lamp.

CAS assay

We used an improved chrome azural S assay (CAS assay), which was modified for applications to seawater samples.[17] A sample solution was mixed with an equal potion of the CAS assay solution (CAS, 2×10^{-4} M; $FeCl_3$, 2×10^{-5} M; hexadecyltrimethylammonium bromide (HDTMA), 1.6×10^{-3} M; 1,4-piperazinediethanesulfonic acid (PIPES), 1.0×10^{-1} M; pH 5.8). After 30 min, the absorbance of the solution was measured at 655 nm. The concentrations of iron(III)-complexing ligands were calculated from the decrements of the absorbance at 655 nm (ε = 105000 M^{-1} cm^{-1}).

Results and Discussion

Choice of reagents and conditions in CLE/ACSV

The reagents and procedures used in the CLE/ACSV determination of iron(III)-binding ligands were considered in view of previous studies of seawater.[1-4,28-30] Two different competitive ligands, 1-nitroso-2-naphthol[1,2] and salicylaldoxime,[3] were commonly used for iron(III) speciation by CLE/ACSV. Since the measurements have been extensively studied for the speciation and reactivity of organically complexed iron(III), 1-nitroso-2-naphthol was selected as the competitive ligand in this study. It is reported that the reduction current of ACSV with 1-nitroso-2-naphthol was enhanced by the addition of oxidizing agents and surfactants, such as H_2O_2, bromate and sodium dodecyl sulfate.[28,29] In choosing the analytical condition, particular emphasis was placed on keeping the chemical forms of the iron(III)-binding ligands throughout the procedure. Therefore, voltammetric scans were carried out at pH 8.0 without oxidizing agents and surfactants, in which the

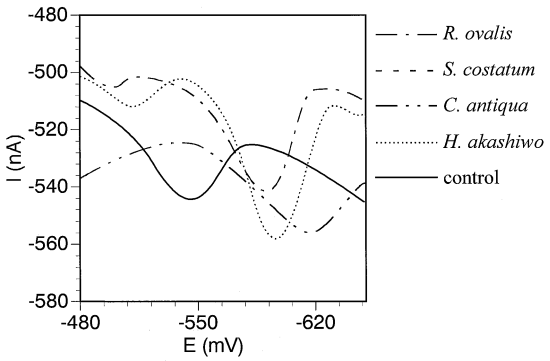

Fig. 1 Interference from exudates of phytoplankton on the determination of iron(III) using ACSV. Voltammetric scans for 30 nM iron(III) and 20 nM EDTA.

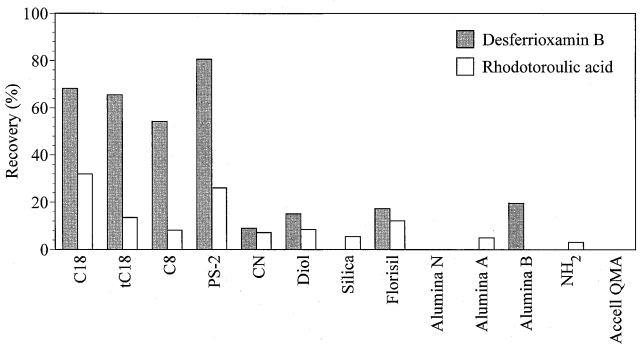

Fig. 2 Solid-phase extraction of two model iron(III)-complexing ligands with Sep-Pac cartridge columns.

measurements of ACSV were sufficiently sensitive to determine the nM levels of iron(III) in sample solutions.

Interferences from exudates of phytoplankton on the determination of ACSV

Figure 1 shows voltammetric scans using ACSV and titrations of CLE/ACSV in the filtrates of phytoplankton cultures. As a control, a modified f/2 medium with 30 nM iron(III) and 20 nM EDTA was used. The iron peak reduction current of the control was observed at –550 mV, which is in agreement with previous studies.[28,29] On the other hand, the peak currents in the phytoplankton filtrates were shifted to a lower potential (Fig. 1). In direct titrations of 20 nM EDTA in the *R. ovalis* and *H. akashiwo* filtrates, the peak currents were independent of the total iron concentrations, and had abnormally high values, even if no iron was added. The filtrates of the *C. antiqua* cultures reduced the sensitivity of the peak currents by at least 70%. These results suggest that exudates from phytoplankton interfere with measurements of the labile iron concentrations using ACSV. It is reported that dissolved organic materials interfere with ACSV measurements, because they lower the surface area of HMDE available for adsorption.[30]

Solid-phase extraction of two model iron(III)-binding ligands

To remove the interferences, extraction of the iron(III)-binding ligands from the phytoplankton cultures was carried out by passage of the samples through Sep-Pak cartridge columns. These commercially produced columns are useful because they are easily handled and their performance is almost constant regardless of manipulations. Various columns for solid-phase

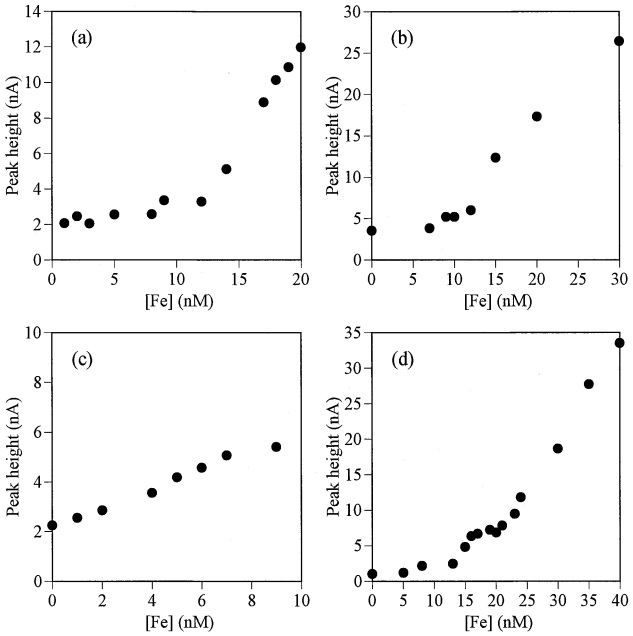

Fig. 3 Titrations of iron(III)-binding ligands in phytoplankton cultures after a sample treatment using a Sep-Pak C18 column. (a) *Chattonella antiqua*, (b) *Heterosigma akashiwo*, (c) *Skeletonema costatum* and (d) *Rhodomonas ovalis*.

extraction were tested concerning the retention capacity with two model ligands, desferrioxamine B and rhodotorulic acid (Fig. 2). A series of columns with hydrophobic absorbents were more effective for the retention of the ligands than any other columns. The extracted compounds in the columns were back-extracted with methanol quantitatively. The C18 and PS-2 columns were relatively effective, of which the recoveries showed 68% and 81% for desferrioxamine B, and 32% and 26% for rhodotorulic acid. This is consistent with previous results of other polymeric resins. The resins of hydrophobic types have been used for the extraction of natural iron(III)-binding ligands in seawater[9] and for the isolation of siderophores from bacterial cultures.[13,14] From the view point of retention efficiency, the C18 and PS-2 cartridge columns were chosen for the separation of unknown iron(III)-binding ligands from the filtrates of phytoplankton cultures. The extraction procedure was repeated three times in order to increase the recoveries of the ligands.

Titrations of iron(III)-binding ligands by CLE/ACSV

During growth experiments, the concentrations of iron(III)-binding ligands were monitored using the CAS assay. In all cultures of *S. costatum*, *C. antiqua*, *H. akashiwo* and *R. ovalis*, the CAS-positive compounds were detected during the exponential growth phase. Titrations were, therefore, carried out using exponential phase cultures after the extraction procedures. The results of iron titrations are presented in Fig. 3. The titration curves obtained from solutions after a sample treatment are clearer than those of untreated solutions. For the *C. antiqua*, *H. akashiwo* and *R. ovalis* cultures, the peak currents were suppressed at low iron addition and increased with iron concentrations at high iron addition. These responses indicate the occurrence of iron(III)-binding ligands in the cultures. For the *S. costatum* culture, the titration curve was the same as that of the control, indicating that none of ligands were detected under the conditions.

An analysis of the titration data was carried out based on the

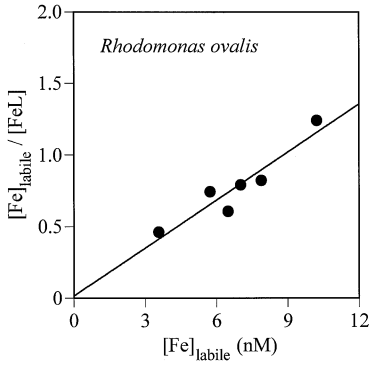

Fig. 4 Typical linearization plot of titration data in Fig. 3.

assumption that iron(III) and the ligands form 1:1 complexes. The ligand concentrations (C_L) and the stability constants (log K'_{FeL}) of the four species of phytoplankton were evaluated by fitting the following equation[2] to experimental measurements:

$$[Fe]_{labile}/[FeL] = [Fe]_{labile}/C_L + (\alpha_{Fe'} + \alpha_{FeNN})/(C_L K'_{FeL}),$$

$$K'_{FeL} = [FeL]/([Fe^{3+}][L']),$$

where [L′] and $[Fe]_{labile}$ are the concentrations of iron(III)-binding ligands (L) not complexed by iron and labile iron, respectively. The α-coefficients ($\alpha_{Fe'}$ and α_{FeNN}) are defined and estimated according to previous work.[1,2] A typical linearization plot for the iron(III)-binding ligands titration from the phytoplankton cultures is shown in Fig. 4, and the results are summarized in Table 2. A plot of $[Fe]_{labile}/[FeL]$ as a function of $[Fe]_{labile}$ is linear, which suggests that iron(III) predominantly forms complexes with a single ligand. This result is similar to previous studies on natural iron-binding ligands in seawater[1-3] and bacterial ligands, such as siderophores.[7,8]

The limit of detection (LOD) of iron(III)-binding ligands by CLE/ACSV titrations depends on the instrumental sensitivity of ACSV and the contamination of iron throughout the procedures. To estimate the LOD, the blank values of the analytical procedure were determined using 200 ml of the modified f/2 medium (Fe(III), 200 nM; no chelating ligands). The LOD of iron(III)-binding ligands was 3.3 nM in sample solutions after the extraction procedures, which was estimated as three-times the standard deviations of the blank values. Therefore, the measurements of CLE/ACSV is at least one order of magnitude more sensitive than that of the CAS assay. The iron(III)-binding ligands can be determined with the titrations by CLE/ACSV at concentrations down to 80 nM or below.

In Table 2, the concentrations of iron(III)-binding ligands obtained from CLE/ACSV are in agreement with those of the CAS assay, except for *H. akashiwo*. The difference in the *H. akashiwo* cultures would be due to the error of the CAS assay. It was reported that measurements of the CAS assay suffer from the interference of organic substances other than the iron(III)-binding ligands.[18] The concentrations of the ligands vary with the phytoplankton cultures, while the stability constants (log K'_{FeL}) are within the range of 25 – 27. The values for log K'_{FeL} in this paper were relatively higher than the reported values of *Emiliania huxleyi*.[16] It is considered that the amount and nature of the iron(III)-binding ligands excreted from phytoplankton is dependent on the species of phytoplankton and their grown conditions.

Table 2 Values for the iron(III)-binding ligands concentrations and the stability constants from phytoplankton cultures

Phytoplankton species	CAS assay C_L[a]/ nmol l^{-1}	CLE/ACSV	
		C_L[a]/ nmol l^{-1}	Stability constants (log K'_{FeL})
Rhodomonas ovalis	400	389 ± 12	26.0 ± 0.2
Chattonella antiqua	290	269 ± 5	26.9 ± 0.6
Heterosigma akashiwo	400	240 ± 18	25.3 ± 0.3
Skeletonema costatum	< 80[b]	< 3.3[b]	—

a. Concentrations of iron(III)-binding ligands in sample solutions after extraction procedures.
b. Limits of detection.

Conclusion

We have reported a method for the detection of iron(III)-binding ligands in phytoplankton cultures by the combination of a solid-extraction technique and electrochemical measurements by CLE/ACSV. The procedures developed in this study could remove interference from exudates of phytoplankton, provide more accurate values than direct measurements by CLE/ACSV, and be applied to the determination of more than 3.3 nmol/l of the ligands with a complexing ability for iron(III). The analytical results of the culture experiments suggested that some coastal species of eukaryotic phytoplankton produce iron(III)-binding ligands and that the excretion of iron(III)-binding ligands might depend on the species of phytoplankton. This analytical method will be available for further investigations to elucidate the molecular structure and character of the iron(III)-binding ligands from phytoplankton.

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