Tolerance and detoxification mechanisms in marine diatom *Phaeodactylum tricornutum* exposed to cadmium

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Abstract.

Tolerance is one of the most important mechanisms for survival. Organisms living in polluted environments with heavy metals develop tolerance to these contaminants; this tolerance has been attributed to the ability to synthesize metal-binding substances or another different mechanism able to mediate metal detoxification. It has been demonstrated that high levels of metals induce the formation of these substances in plants, algae, and some fungi (phytochelatins), which are a protective mechanism against the toxicity of heavy metals. In this study we have addressed the capacity of the microalga *Phaeodactylum tricornutum* to produce metal-binding pep tides in response to stress induced by cadmium. Liquid chromatography using biogel P-60, thiol analysis in chromatographic fractions, and capillary electrophoresis provided evidence of phytochelatins production in this alga.

Contamination of rivers and estuaries with heavy metals has been documented in numerous surveys. The toxic effects of high concentrations of these trace metals on aquatic organisms, including algae, have also been the subject of an extensive literature (Whitton and Say 1975; Vymazal 1987). Heavy metals are important pollutants in aquatic environments with adverse effects on the organisms.

Tolerance is one of the most important mechanisms for survival. A variety of tolerance mechanisms against cytotoxic effects of heavy metals have been described (Maeda and Sakaguchi 1990). Thus, many organisms respond to this effect by synthesizing metal chelating-proteins or peptides. In plants, algae, and some fungi, these peptides were designated as: phytochelatins (PC), cadystins, Cd^{2+}-binding peptides (Cd-BP), or y-glutamyl peptides (Steffens 1990). Because of their ability to bind heavy metal ions, these molecules are considered to play a role in cellular metal homeostasis and metal detoxification.

Phytochelatins have the general structure $(\text{y-Glu-Cys})_n\text{-Gly}$, where *n* can range from 2 to 11 depending on the species and the conditions of their induction. In vascular plants, the biosynthesis of phytochelatins is catalyzed by a specific y-glutamylcysteine-dipeptidyl transpeptidase, called phytochelatin synthase, which is activated in the presence of metal ions and uses glutathion (GSH) as a substrate. An additional component of these molecular complexes is acidlabile sulfur (Steffens et al. 1986). Incorporation of sulfide in PC-metal complexes can result in the formation of metal-S crystallites, which might be the storage form of metal (Ortiz et al. 1992).

Evidence that microalgal Cd tolerance is achieved by a mechanism that includes binding of induced polypeptidesto this metal ion was first obtained with *Euglena gracilis* (Gingrich et al. 1986).

Cadmium has been shown to increase sulfate reduction in plants (Nussbaum et al. 1988). Incorporation of acidlabile sulfur into Cd-peptide complexes increases the metal-binding affinity. The relevance of the presence of sulfide in PC-metal complexes to metal detoxification is substantiated by the observation that mutants of *Schizosaccharomyces pombe* that produce only PC-Cd complexes without sulfide are hypersensitive to Cd (Mutoh and Hayashi 1988).

In the present study, the resistence of the marine diatom *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae) to cadmium by means of phytochelatins and acid-labile sulfur was demonstrated.

Materials and methods

Algal culture

P. tricornutum cells were cultured in seawater with modified ALGAL-1 medium without EDTA (Herrero et al. 1991) at 18 ± 1°C with a dark-light cycle of 12: 12 h (68 µE/m^2/s*)*. Algae were cultured in batch conditions using 250-ml bottles in which cells were exposed to 200 µM $CdCl_2$ for 48 h during the logarithmic growth period. Control cultures under the same conditions but without $CdCl_2$ were also included. Experiments were carried out in triplicate. Growth was monitored by counting cells in an hemocytometer (Neubauer).

Preparation of cells extracts

Algal cells were removed from culture media by centrifugation (3500 *g* for 10 min) and washed once in natural mewl free seawater. The pellet of algal cells was then resuspended in degassed borate buffer (100 mM, pH 8.6). and the cells were homogenized with an ultrasonic cell disrupter. The insoluble cell debris from the homogenate was removed by centrifugation at 13,000g for 10 min and 30,000g for 30 min. The cleared supernatant was applied to BioGel P-60 column.

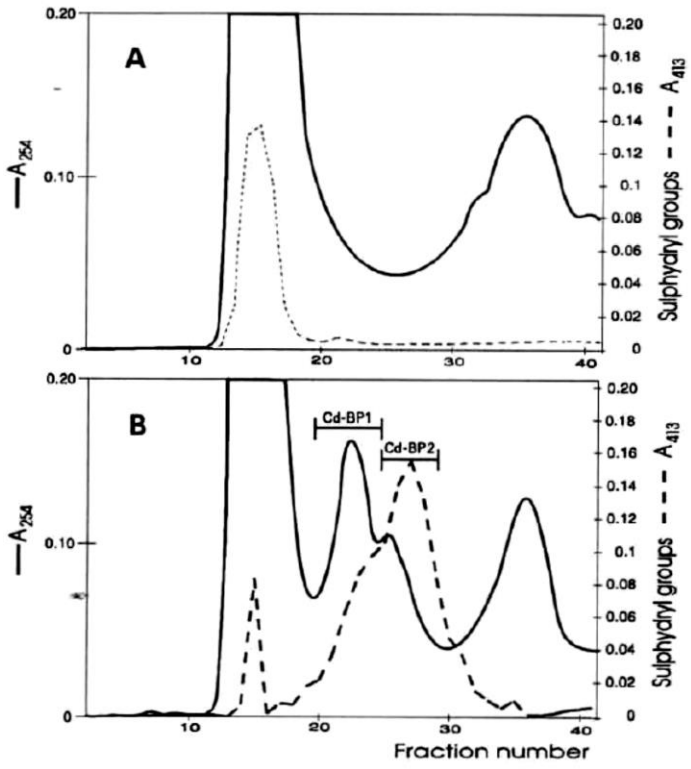

Fig. 1. Elution profiles of gel permeation chromatography on BioGel P-60 of untreated **(A)** and Cd-treated **(B)** *P. tricornutum* cells. Solid line: optical density at 254 nm; dashed line: sulfhydryl groups.

Purification of Cd-BP complexes

To study the formation of the Cd-BP complexes, the cleared supernatant was analyzed by gel permeation chromatography. A column (2.5 x 50 cm) of BioGel P-60 (medium, Bio-Rad Laboratories) previously equilibrated with the same borate buffer (100 mM, pH 8.6) was used. The extract was eluted at room temperature (25°C) with a flow rate of 20 ml/h. The absorption of the eluate was monitored at 254 nm and fractions of 5 ml were collected.

Detection of thiol-rich compounds

Thiol-rich compounds in each fraction were detected by the method of Ellman (1959). The sample was mixed with an equal volume of a solution containing 2 mM 5,5'-dithiobis(2-nitrobenzoic) acid and 0.1 M degassed potassium phosphate buffer, pH 8. After 10 min of reaction, the absorption was recorded at 413 nm against a blank sample with a UV/VIS spectrophotometer.

Analysis of Cd-thiol-rich complexes

Fractions from the BioGel P-60 column containing thiols were pooled and the volume reduced by ultrafiltration in a Macrosep system (Filtron 3K).

The concentrated samples were analysed by micellar electrokinetic capillary chromatography (MECC). Samples were analysed using Quanta 4000 capillary electrophoresis system (Waters) with fused-silica capillary.

The electrolyte used was 100 mM borate buffer, pH 8.6 and containing 1% sodium dodecyl sulfate (SDS). Samples were loaded by hydrostatic injection for 15 s and separations were performed at 10 KV and 25°C. Separated components were detected at 214 nm.

Results and discussion

The effect of different concentrations of cadmium on the growth of *P. tricornutum* was previously assayed to define meta; concentration inhibiting cell growth but not cell viability. This concentration was used for the assays.

The analysis of the elution profiles of gel permeation chromatography on BioGel P-60 of crude extracts (Fig. I) from untreated (A) and Cd-exposed cells (B) showed two new peaks in the extracts from Cd-exposed cells. These peaks are the cadmium-induced

complexes, and they are called Cd-BP1 and Cd-BP2. These new molecules are similar to the complexes formed in the fission yeast *Schizosaccharomyces pombe* exposed to cadmium (Mutoh and Hayashi 1988). Because Cd-BP1s have greater Stokes radii than CdBP2s they can be separated by gel permeation. Cd-BP1 contains inorganic sulfur in addition to phytochelatins and cadmium. Cd-BP2 is composed of phytochelatins and cadmium. Thus, two populations of metal-peptide complexes differ in the content of bound sulfide ions (Mutoh and Hayashi 1988).

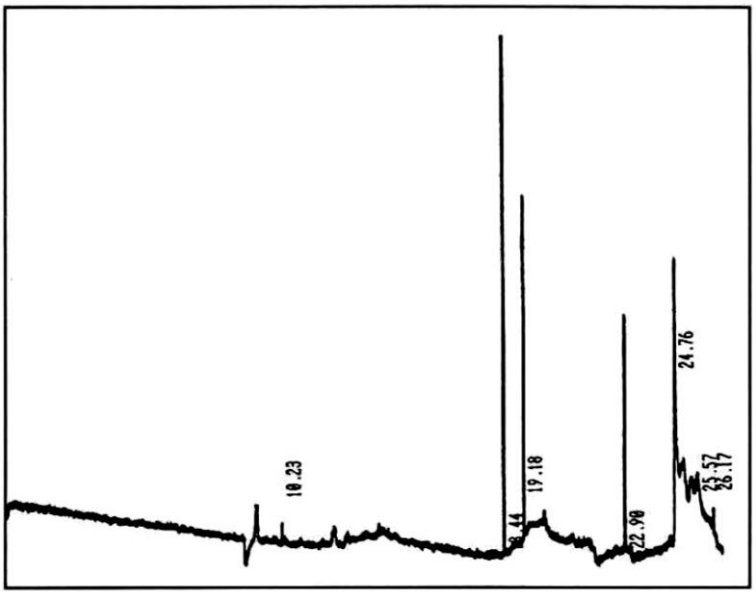

Fig. 2. Micellar electrokinetic capillary chromatography of Cd-BP1 fractions. Conditions: 100 mM Borate buffer with 1% SDS, pH 8.6, and detection system was UV at 214 nm.

Sulfhydryl (SH) measurements (Fig. 1) show that Cd- BPI and Cd-BP2 contain the highest levels of SH. The high sulfide level in the complexes appears to be a response to high concentrations of cadmium in plants and may be important in the tolerance to heavy metal stress, since sulfide increases the metal-binding affinity and stoichiometry of the complex (Reese and Winge 1988). Incorporation of sulfide ions in the complex and the resulting higher stability and metal-binding capacity may increase the effectiveness of these peptides complexes as a mechanism for sequestration of toxic metals. In *P. tricornutum* cells the high level of sulfhydryl groups found probably indicates a similar mechanism of protection, although the maximum level of sulfuydryl groups is not coincident with the two complexes, probably because in the complexes the sulfhydryl groups are bound to cadmium, and the reaction with EIIman's reagent is weaker.

Electropherogram for Cd-BP1 fractions of *P. tricornutum* is shown in Fig. 2. Results show that Cd-BP1 is composed of at least four different chains of phytochelatins with

different numbers of residues (y-Glu-Cys). These capillary electrophoresis results are similar to reverse-phase HPLC results (Gekeler et al. 1988), but the elution order of the different chains of phytochelatins by micellar electrokinetic capillary chromatography is still unknown. We can designate these peptides as PC_2 PC_3 PC_4 and PC_5 as they are obtained in HPLC. More studies about this procedure will permit us to increase the peak resolution and determine the elution order.

Because *P. tricornutum* produce Cd-binding polypeptides, its high metal tolerance may be attributed to a mechanism that includes the production of these polypeptides. Thus, phytochelatins or Cd-binding polypeptides appear to be the primary metal-binding polypeptides in *P tricornutum* cells. These molecules are involved in metal homeostasis and metal detoxification. They are heterogeneous complexes in their peptide composition and contain labile sulfur in addition to heavy metals. In these molecules cadmium is sequestered, preventing the presence of free cadmium in the cytoplasm, reducing its toxicity.

References

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70-77

Herrero C. Cid A, Fabregas J, Abalde J (1991) Yields in biomass and chemical constituents of four commercially important marine microalgae with different culture media. Aquacult Eng 10:99-110

Gekeler W, Grill E, Winnacker E-L, Zenk MH (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. Arch Microbiol 150:197-202

Gingrich DJ, Weber ON, Shaw CF, Garvey JS, Petering OH (1986) Characterization of highly negative and labile binding protein induced in *Euglena gracilis* by cadmium. Envir Hlth Persp 65:77- 85

Maeda S, Sakaguchi T (1990) Accumulation and detoxification of toxic metal elements by algae. In: Akatsuka I (ed) . Introduction to applied phycology. pp 109- 136

Mutoh N, Hayashi Y (1988) Isolation of mutants of *Schizosaccharomyces pombe* unable to synthesize cadystin, small cadmium-binding peptides. Biochem Biophys Res Commun 151 :32-39

Nussbaurn S, Schmutz D, Brunold C (1988) Regulation of assimilatory sulfate reduction by cadmium in *Zea mays* L. Plant Physiol 88: 1407-1410

Ortiz OF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow OW (1992). Heavy metal tolerance in the fission yeast requires an ATP-binding cassette- type vacuolar membrane transporter. EMBO J 11:3491 - 3499

Reese RN. Winge OR (1988) Sulfide stabilization of cadmium-g-glutamyl peptide complex of *Schizosaccharomyces pombe.* J Bioi Chem 263: 12832- 12835

Steffens JC (1990) The heavy metal-binding peptides of plants. Annu Rev Plant Mol Bioi 41:553-575

Steffens JC. Hunt DG. Williams BG (1986) Accumulation of non-protein metal-binding polypeptides (glutamyl-cysteinyl)n-glycine in selected cadmium- resistant tomato cells. J Biol Chem 263 : 13879- 13882

Vymazal J (1987) Toxicity and accumulation of cadmium with respect to algae and cyanobacteri a: A review. Toxicity Assess 2:387-415

Whitton BA. Say PJ (1975) Heavy metals. In: Whitton BA (cd). River ecology. Blackwell. Oxford. pp 286--311