Bull. Environ. Contam. Toxicol. (2001) 66:357–364

DOI: 10.1007/s00128-001-0013-2

Bulletin of Environmental Contamination and Toxicology

Synthesis Pattern of an Hg-Binding Protein in *Acetabularia calyculus* During Short-Term Exposure to Mercury

E. M. García, R. E. Reyes

Department of Organisms Biology, Simon Bolivar University, Sartenejas, Baruta, Apto. 89000, Caracas 1080, Venezuela

Received: 30 June 1999/Accepted: 18 December 2000

Algae and aquatic macrophytes are capable of accumulating heavy metals up to concentrations several orders of magnitude higher than those existing in their surrounding environment. In fact, algae have been used to extract heavy metals from water and to enriching and recycling valuable metals (Gekeler et al., 1988).

This ability to accumulate heavy metals has been associated with the induction of metal-binding proteins (Tripathi et al., 1996) which is enhanced when organisms are exposed to high concentrations of a wide variety of metals (Grill et al., 1985;1987). Heavy metals have been shown to induce the synthesis of these peptides, also known as phytochelatins. This cellular response depends on the particular metal and its concentration (Grill et al., 1985; Steffens, 1990). Today, we know that under normal environmental concentrations, these proteins are part of the homeostasis of metals essential to life (Vogely and Wagner, 1990).

Mercury is one of the most toxic metals in the aquatic environment. However, very little is known about its effect on benthic marine algae. *Acetabularia calyculus* is a benthic unicellular alga that possesses the ability to bioconcentrating high amounts of Hg and to simultaneously synthesizing a mercury-binding protein (Garcia 1993; Garcia and Reyes, 1996). In this paper we study the induction of metal-binding proteins as a function of the bioconcentration factor and the time of exposure to high Hg concentrations of *Acetabularia calyculus*.

MATERIALS AND METHODS

Cells of *Acetabularia calyculus* were cultivated in artificial seawater (referred as MS) (Garcia, 1993) at 23-25^{0}C (light-dark 12hr-12hr, about 3-4 W/m^2) in growth chamber.

Correspondence to: E. M. García

Long whole cells and anucleated cells (3.8 - 4cm) were selected to perform the contamination assays. For treatments, algae (250 cells, approximately 15-g fresh weight) were exposed to three concentrations (1, 5 and 10 mg/L) of Hg as mercuric chloride ($HgCl_2$) prepared in MS, during periods of time of 24, 72 and 168 hr. Algae cultured without Hg served as controls. Each treatment was performed three times for one set of experiments. The whole set of experiments was performed twice.

After treatment, whole cells were cut in two sections: rhizoid (or nucleus) was discarded. Stalks (or cytoplasm) were used for further measurements (i.e. Hg concentration, protein extraction). We report only the cytoplasm section. For anucleated cells, the section referred as stalk was separated from the rhizoid 24 h before treatment (Garcia, 1993).

Analysis of total Hg was carried out following the procedure EPA (1976) using a Perkin Elmer Atomic Absorption Spectrophotometer (Model 2380) (detection limit – 0.002 μg/L). The validity of these analytical methods was compared to EPA's standard reference. Differences of Hg concentration due to evaporation and adsorption were measured in the MS at the beginning and at the end of each experiment. All readings of Hg concentration in the control experiments were below the detection limit (0.002 μg/L). The bioconcentration factor (BCF = ratio [Hg in the cell] / [Hg in the external seawater]) was determined in each treatment.

For the analysis of protein synthesis, proteins were extracted according to Garcia and Reyes (1996). Two fractions of protein, referred as nuclear protein fraction and cytoplasmic protein fraction, were obtained from nucleated cells. Only one fraction, anucleated cytoplasm protein fraction, was obtained from anucleated cells.

Bio-Rad Mini-Protein II slab gel was used for SDS-electrophoresis. The stacking gel contained 4% acrylamide-bisacrylamide and separation gel contained 15% acrylamide. Electrophoresis was carried out at 37 mA/plaque for 40 min. Gels were stained with silver nitrate (Scopes, 1988). The protein content was determined by a Bio-Rad protein assay (Bradford, 1976). Samples from treated cells and control cells were run under similar conditions.

Gel densitometry was done using an Imaging Densitometer Bio-Rad (Model G5-690) and its values were determined by the Bio-Rad Molecular Analysis program. Treatments for bioconcentration factors were compared by analysis of variance (ANOVA) and the significance was defined as $p <= 0.05$ (Remington and Schork, 1979).

RESULTS AND DISCUSSION

Table 1. Densitometry readings of Hg bands present in the electrophoretic Polyacrylamide gels ; bioconcentration factor of 3 and 7 days in whole cells.

Treatment	Bioconcentration Factor Ratio (Hg Algae/(Hg water) (Whole cells)		Exposure Time Densitometry Reading (VolXD.O.Xmm^2) (Whole cells)	
$HgCl_2$ (mg/L)	3d	7d	3d	7d
Control	>0.002	>0.002	8.40±0.03	5.02±0.02
1	40.5±0.85	37.5±1.25	23.41±0.01	12.46±0.04
5	52.3±0.85	53.5±1.20	28.74±0.02	23.24±0.01
10	74.4±1.77	64.6±1.11	39.65±0.01	1.17±0.01

Data are means ± SD (n=9, $p < 0.05$). SD did not exceed 2% of the mean for nine densitometry readings.

Table 2. Densitometry readings of Hg bands present in the electrophoretic Polyacrylamide gels and Bioconcentration Factor of 3 and 7 days in anucleated cells.

Treatment	Bioconcentration Factor. Ratio (Hg Algae/(Hg water) (Anucleated cells)		Exposure Time Densitometry Reading (VolXD.O.Xmm^2) (Anucleated cells)	
$HgCl_2$ (mg/L)	3d	7d	3d	7d
Control	>0.002	>0.002	11.61±0.01	6.17±0.08
1	40.3±0.89	34.5±1.28	22.45±0.02	11.24±0.01
5	50.6±1.06	54.5±1.40	24.59±0.01	26.18±0.01
10	73.5±1.45	68.3±1.67	43.45±0.01	4.95±0.01

Data are means ± SD (n=9, $p < 0.05$). SD did not exceed 2% of the mean of nine densitometry readings.

Densitometry readings of samples (Table 1 and 2) indicate that after 3 days of exposure the amount of the metal-induced band (see arrow in Fig.1) increased at $HgCl_2$ concentrations of 1 - 10 mg/L. This band was shown to be a mercury-binding protein with all features (SH-groups and capacity to bound Hg) commonly found in a metal- binding protein (Garcia and Reyes, 1998). Electrophoretic profiles of individual samples from each treatment are shown in Figs.1 and 2. In these treatments, a dose-dependent increase of synthesis of

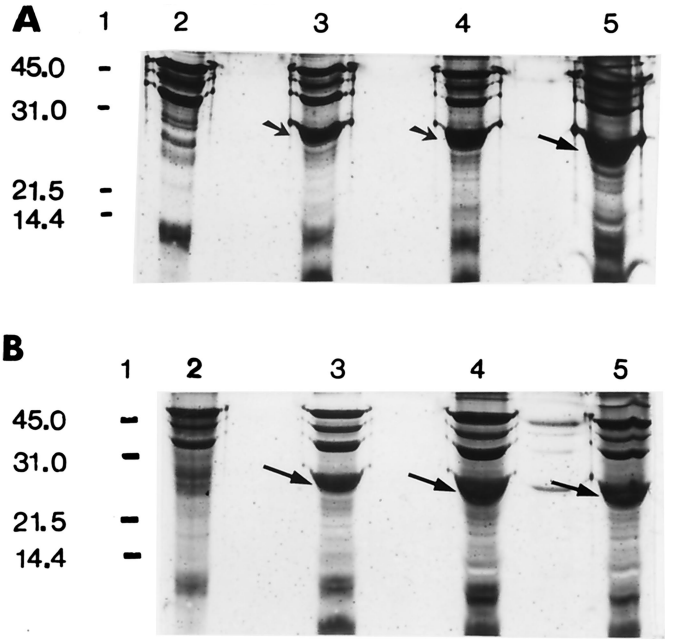

Figure 1. Protein SDS-gel electrophoresis. Three days treatment: (**A**) Whole cells. (**B**) Anucleated cells. From left to right: 1. Molecular weight markers. 2. Control cells. 3. Cells treated with 1 mg/LHg. 4. Cells treated with 5 mg/LHg. 5. Cells treated with 10mg/LHg.

protein is observed in both, whole and anucleated cells (Fig.1). However, very low levels of protein are found in the control cells. The maximum induced response was achieved when treatments used 10mg/L of $HgCl_2$.

The Hg-induced protein exhibited very similar electrophoretic patterns of synthesis in both whole and anucleated cells (either at 3-days and 7-days treatments), indicating that, under experimental conditions, its appearance is not determined by the presence of the nucleus (Fig.1B). This rapid production of protein upon exposure to Hg in both cell types indicates that the mechanism for short-term synthesis response is controlled and expressed by a cytoplasmic pathway. Long term, gene-regulated response, as proposed for Cu-tolerant plants, could also be taking place, as a complementary detoxification mechanism (De Miranda et al., 1990).

The bioconcentration of Hg increased significantly ($p < 0.01$) as the Hg concentration and duration of exposure increased (Table 1 and 2). At the highest tested concentration (10mg/L), the BCF for whole and anucleated cells was 74.4 ± 1.20 and 73.5 ± 1.45, respectively, over 3 days.

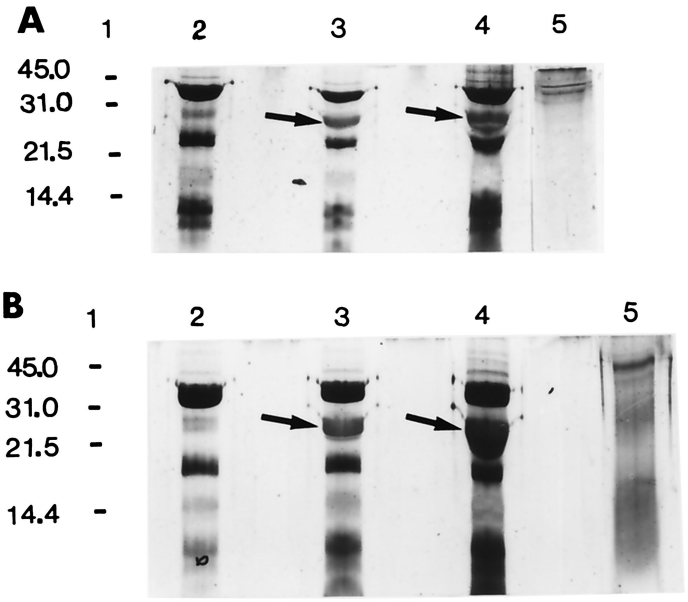

Figure 2. Protein SDS-gel electrophoresis. Seven days treatment: (**A**) Whole cells. (**B**) Anucleated cells. From left to right: 1. Molecular weight marker. 2. Control cells. 3. Cells treated with 1 mg/L.Hg 4. Cells treated with 5 mg/L.Hg 5. Cells treated with 10mg/L.Hg.

(Tables 1 and 2). In short-term incubations, the ability to uptake Hg does not seems to depend on the nucleus.

These results show that: (1) The environmental Hg concentration and the induction of protein synthesis are directly related in 3-days treatments, and (2) The induction response is similar in both whole and anucleated cells.

Qualitative differences of overall protein banding are noticed after comparing 3-days and 7-days treatments (Fig. 2), using 1 - 10 mg/L $HgCl_2$. The intensity of all the protein bands from 7-days treatment, and that of the Hg-induced band, were significantly reduced. The reduction of band intensity was the strongest (99% to 96%) in treatments using the highest Hg concentration (10 mg/L) in 7-days treatment. This difference between 7-days and 3-days treatments may result from the inability to synthesize the polypeptide during long-term exposure, owing to the cytotoxic effects of high concentrations of Hg. This interpretation should not be surprising because: (a) the overall banding pattern in this treatment is less intense, specially at high concentrations of Hg (10mg/L), and (b) in all treatments, high molecular weight proteins disappear, suggesting that stress is affecting all the cells under this treatment. Moreover, visual damage (loose of chlorophyll, plasmolysis and bleaching) was detected in external features of both types of cells (whole and anucleated) when exposed to 10 mg/L of Hg. Similar toxic effects have been observed in aquatic macrophytes and algae exposed to heavy metals

such as cadmium and copper (Tripathi et al, 1996; Gupta and Chandra, 1996; Morelli and Pratesi, 1997).

In 7-days incubation experiments, the densitometry values of the Hg-induced bands and the BCF were significantly lower ($p < 0.05$ for the BCF) than those obtained in 3-days treatment (Fig.2). However, in the 7-days treatment, the cells are still capable of effectively accumulating Hg, as it is demonstrated by the high levels found of intracellular Hg (BCF = 64.6 ± 1.1 in whole cells, BCF = 68.3 ± 0.67 in anucleated cells using 10mg/L). This result suggests that *Acetabularia calyculus* is able to bioconcentrate Hg even in the absence of the Hg-induced polypeptide, which seems to play a role in the early response of the alga to Hg exposure.

According to these results, the activation of the Hg-binding protein could be an early response to external Hg concentration that depends on the intracellular metal concentration and allocation in the different cell compartments (Grill et al 1988). These responses do not directly depend on the nucleus since the same pattern was observed in both whole and anucleated cells.

However, during long periods of exposure , Hg-induced polypeptides do not seem to be related directly with the accumulation of Hg by the cells. Actually, the reduced production of the Hg-binding protein under these conditions, suggests that other detoxifying (and/or storing) mechanisms are taking place. In fact, high intracellular concentrations of Hg could be the result of the accumulation of Hg in the vacuole and the degradation of the Hg-binding protein that follows.

Similar results, reflecting a decrease in phytochelatin production as a response to high Cu concentrations have been reported by Ahner and Morel (1995) in marine phytoplankton. In their experiments, a rapid phytochelatin production was followed by either its rapid degradation to glutathione, to amino acids, or its excretion.

The expression of the Hg-binding protein is affected by the length of the period in which the alga is exposed to Hg. The results obtained in this study suggest that the tolerance of *Acetabularia calyculus* to Hg is proportional, in short-term exposure conditions, to the production of Hg-binding protein, which is necessary to sequester and bioconcentrate the intracellular metal. On the other hand, during long-term exposure to Hg, a trade-off seems to occur between the production of the Hg-binding protein and the storage and accumulation of Hg. Further research is in progress in order to untangle the mechanisms that control this response shift.

Acknowledgments. This project was supported by CONICIT Grant S1-95000705. The authors gratefully acknowledge Dr. Gabriel Farache, Dr. Roberto Cipriani, and Ms. Miriam Barbosa for helpful suggestions and technical assistance.

REFERENCES.

Ahner BA, Morel FM, (1995) Phytochelatin production in marine algae. 2. Induction by various metals. Limmol Oceanogr 40:658-665

Bradford M,(1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72:248-254

De Miranda JR, Thomas, M.A, Thurman D.A, Tomsett A.B, (1990) Methallothionein genes from the flowering plant *Mimilus guttatus*. FEBS Lett 260:277-280.

Environmental Protection Agency (1976). Manual of methods for chemical analysis of water and wastes (Springfield) p. 1183.

Garcia EM, Reyes RE, (1998) Induction of mercury-binding peptide in whole cells and anucleated cells of Acetabularia calyculus. Toxicol Environ Chem 67 189-196

Garcia EM, Reyes RE, (1996) Bioconcentration of mercury in *Acetabularia calyculus*: evidence of a polypeptide in whole cells and anucleated cells. Toxicol Environ Chem 55:11-18

Garcia EM, (1993) The ability of the unicellular giant alga *Acetabularia sp.* to bioconcentrate aquatic mercury in whole cells and anucleated cells. Toxicol Environ Chem 39:29-35

Gekeler W, Grill E, Ludwing E, Zenk M, (1988) Algae sequester heavy metals via synthesis of phytochelatins complexes. Arch Microbiol 150:197-202

Grill E, Winnaker E, Zenk M, (1985) Phytochelatins: the principal heavy metal-complexing peptides in higher plants. Science 230:574-576

Grill E, Winnaker E, Zenk M, (1987) Phytochelatins, a class of heavy- metal-peptides from plants, are functionally analogous to metallothioneins. Proc Natl Acad Sci 84:439-443.

Gupta M, Chandra P, (1996) Bioaccumulation and Physiological changes in *Hydrilla verticillata* (IF) Role in response to mercury. Bull Environ Contam Toxicol 56:319-326

Morelli E, Pratesi E, (1997) Production of phytochelatins in the marine diatom *Phaeodactylum tricornutum* in response to copper and cadmium exposure, Bull Environ Contam Toxicol 59:657-664

Remington R, Schork M, (1979) Estadistica Biometrica y Sanitaria. Dossat Edit, Spain.

Scopes R, (1988) Protein Purification: Principles and Practice. Springer-Verlag, New York. p. 380.

Steffens SJ, (1990) The heavy metal-binding peptides of plants, Ann Rev Plant Physiol & Plant Mol Biol 41:553-575

Tripathi RD, Rai UN, Gupta M, Chandra P, (1996) Induction of phytochelatins in Hydrilla verticillata(I.F) Role under cadmium stress, Bull Environ Contam Toxicol 56:505-512

Vogeli R and Wagner G. (1990) Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves. Plant Physiol 92:1086-1093.