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Cadmium-Binding Peptide in Whole Cells of *Acetabularia calyculus*

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Toxicological studies on the unicellular algae *Acetabularia calyculus* have demonstrated that it is capable of bioconcentrating of mercury (Garcia, 1993) and cadmium (Reyes et al, 2001) up to large intracellular concentrations, and directly proportional to the concentration of metals in the environment. In *Acetabularia*, the bioconcentration of Hg was related to the induced synthesis of metal-binding proteins, or phytochelatins. This synthesis occurs as a response to the exposure of the algae to heavy metals (Garcia and Reyes, 1998, 2001). It is believed that this response is involved in the modulation of intracellular concentration of metals (Garcia and Reyes, 2001).

In autotrophic organisms, the induction of phytochelatins, or metallothioneins type III, is one of the main indicators of the exposure to heavy metals (Grill et al., 1987, Cobbett, 2000). These proteins are of low molecular weight and are rich in sulphydryl groups. The biosynthetic origin of metal-binding proteins is related to the synthesis of glutathione, and their function has been extensively demonstrated (Rauser, 1990).

In the present study we evaluate the synthesis and/or modifications of the metal-binding proteins of *Acetabularia calyculus* induced by short-term exposure to cadmium.

MATERIALS AND METHODS

Cells of *Acetabularia calyculus* were cultivated according to Garcia (1993). We selected vegetative cells 4 cm long, with caps.

Whole algae were exposed to a 20mg/l $CdCl_2$ solution in sterile seawater, for three days (Shepard, 1976). All assays and controls were performed in triplicate. At the end of the period of exposure, algae were rinsed twice with seawater/EDTA 5mM (ethylene diamine tetra-acetic acid) for 5 min to remove the excess of metal adhered to cellular walls. Then, the nucleus of each cell was removed for protein extraction. Cells were stored frozen at –20°C until further processing.

Thirty (30) stalks per test (about 10g of wet weight) were homogenized in 2 ml of Tris-HCl Buffer 25mM NaCl, 0.15 mM, DTT (dithiothreitol) 5 mM and PMSF

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(phenylmethanesulfonyl fluoride) 1 mM, pH 8.3. The homogenate was centrifuged at 12,000 g. and 4°C for 15 min in a Sorvall Super-Speed RC-26 Plus centrifuge. The supernatant was centrifuged at 4°C and 100.000g for 30 min in a Sorvall ultracentrifuge. We used the method of Bradford (1976), using BSA (bovine serum albumin) as a standard, to determine the total protein content of the soluble extract.

The soluble extract (70µg/ml of protein) was loaded in a Sephadex G-75 column (Sigma 1.5x32 cm) in Tris-HCl buffer 25 mM, NaCl 0.15 M pH 8.3, calibrated with the molecular weights. The flow speed was 1ml/min. Fractions of 2 ml were analysed at 254nm of absorbance in a Spectrophotometer Shimazu U.V. From each fraction, 100µl were taken for titration of the sulphydryl group, according to Séller et al (1987), and using Ellman's (1959) agent, DTNB (5`5dithiobis-2-nitrobenzoic acid). The absorbance at 412nm was measured using reduced glutathione (1mg/ml) as standard (Sigma, Co). We used a student t-test to detect significant differences ($p \leq 0.05$) in sulphydryl content between contaminated algae and their control.

To determine total thiols (TT), 1 gr of cells was homogenized in sodium phosphate buffer (Na_2HPO_4) pH 7.5, and centrifuged at 12,000g during 5 min. Thiols were quantified according to the reaction of Ellman (1959). For non-protein thiols (TNP) determination 1 gr of cells was homogenized in 5-sulphosalicilic acid (5%) following the same procedure for TT. Protein thiols (TP) were calculated subtracting the values, TT-TNP=TP (Séller et al, 1987). All the tests were made in triplicate and glutathione was used as standard. Student-t tests were used to determine if there was a significant difference in the contents of thiol between algae exposed to cadmium and their control.

To determine cadmium concentration, the content of each pair of column fractions was pooled and digested with 0.2% of HCl during 6 hours at 60°C, following Greenberg, et al. (1992). After digestion, the concentration of cadmium was determined using atomic absorption spectrophotometry in a Perkin Elmer Model 2380 flame spectrophotometer, and using certified cadmium (Sigma) as standard. The limit of detection of the technique was 0.003mg/l. The concentration of cadmium in water at the beginning and at the end of the bioassays indicated that losses were always less than 1%. All the concentration values of cadmium are shown as the average of ten (10) readings.

RESULTS AND DISCUSSION

Peaks of absorbance at 254 nm, with a maximum between 65-1.3 KD, were found in the gel filtration chromatography of total proteins in samples of *Acetabularia calyculus* not exposed to cadmium (Figure 1A). Extracts obtained from algae exposed to cadmium showed a similar pattern of absorbance to that observed in the control, with no major peak shifts. However, a peak in the region of 14 KD and not observed in the control (Figure 1A), was present in the elution profile of contaminated algae (Figure 1 B).

The distribution of the sulphydryl groups showed a pattern similar to that found in the protein absorbance. However, a noticeable increase in the mean values of the sulphydryl groups was found in the fractions of algae exposed to cadmium, in relation to the control. In Figure 2, the peaks are referred to as **I** ($t_{0.05,8}$ = 3.0, $p<0.01$), **II** ($t_{0.05,3}$ = 4.2, $p<0.005$), and **IV** ($t_{0.05,7}$ = 3.1, $p< 0.0001$).

The content of cadmium associated with polypeptide fractions from total protein, revealed an association of this metal (73.89%) with the fraction corresponding to peptides of molecular weight between 2.5 and 1.5 KD (peak **IV**). The remaining intracellular cadmium (25.91%) was associated with the fractions of molecular weight between 60 to 12.4 KD (Table1), corresponding to peaks **I** and **II**.

Concentrations of TT and TP in the soluble extract of the algae exposed to cadmium were higher than those found in the control. However, this difference was not statistically significant (TT: $t_{0.05,4}$ = -2.8; $p>0.05$; TP: $t_{0.05,4}$ = -0.9; $p>0.05$). On the other hand, TNP induced after cadmium exposure (Table 2), exhibited a significant increment ($t_{0.05,4}$ = 4.2; $p<0.05$).

Heavy metal detoxifying mechanisms in plants and fungi are well known (Rauser 1999). Some of them involve the association between low molecular weight phytochelatins and metallic ions. The role of these proteins seems to be related to the regulation of endogenous metals, such as Cu and Zn, as well as to the control of the toxicity of pollutants in the organism, such as cadmium and mercury (Gekeler et al. 1988; Grill et al. 1987).

Previous studies with *Acetabularia calyculus* have shown that a metal-binding protein with a large content of sulphydryl groups is induced in the presence of Hg (Garcia and Reyes 1998, 2001). The results obtained here with whole cells of *Acetabularia calyculus* demonstrate that short-term exposure to cadmium induces the appearance of a peptide of 14 KD in the treated algae that is absent in the control. There was a concomitant significant increase of sulphydryl groups in the constitutive peptides of the algae, particularly in those of very low molecular weight. Furthermore, most of the intracellular cadmium (70%) was associated with these low molecular weight peptides (1.8 KD). As found in metal-binding proteins and metallothioneins from several organisms (Hamer, 1986), the association metal-peptides is probably related to the large content of sulphydryl groups in the proteins.

In previous studies, these polypeptides seem to be present in control cells as well but in low concentrations and in a constitutive manner (Garcia and Reyes, 2001). According to our results, the 14 KD peptide is not a constitutive protein of *Acetabularia* cells, since it was absent from the control extract. Its synthesis seems to be induced by the exposure of cells to cadmium. This protein posses a high content of sulphydryl groups and for this reason a substantial fraction of the intracellular cadmium was found associated to it.

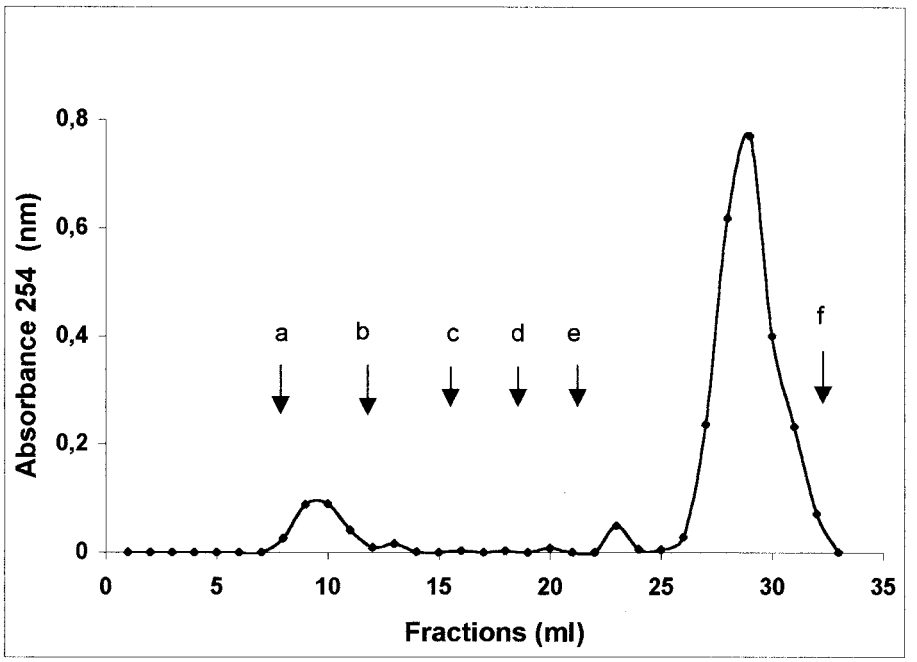

Figure 1A. Gel filtration chromatography (Shephadex G-75) of the soluble protein extract from control cells. Arrows: molecular weight standards: **a**: blue dextran, **b**: 66 KD, **c**: 29 KD, **d**: 12.4 KD, **e**: 6.5 KD **f**: 1.34 KD

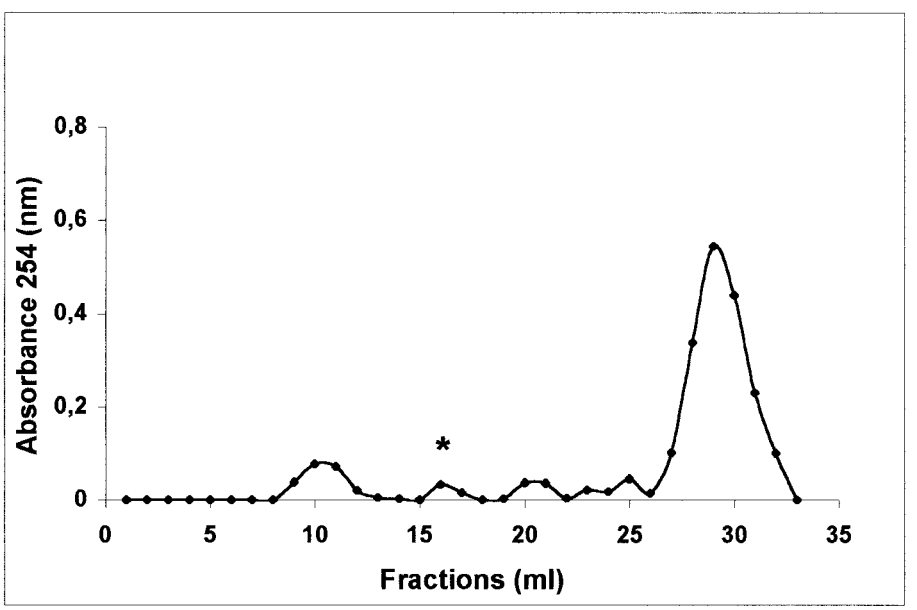

Figure 1B. Gel filtration chromatography (Shephadex G-75) of soluble protein extract from cells exposed to cadmium. *: 14 KD peptide.

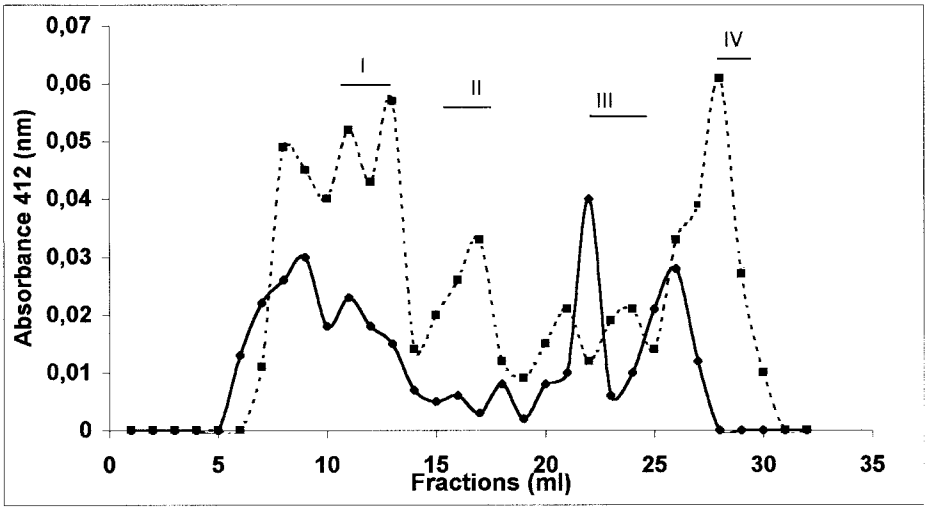

Figure 2. Profile of thiol (SH Groups) from the soluble extract of control cells (solid line) and from cells exposed to cadmium (dotted line).

In several organisms, intracellular cadmium has been found associated with peptides of low (8.0 – 9.0 KD) and very low (1.8 – 3.0 KD) molecular weight (Murasugi et al 1981; Grill et al 1985).

In autotrophic organisms, cadmium is associated to compounds of molecular weight lower than 13 KD. This association is also found in *Acetabularia* (this study), in the diatom *Phaeodactylum tricornutum* (Morelli and Pratessi, 1997), and in many other macroalgae (Hu and Wu, 1998). In all of them, cadmium is mostly found associated with peptides of molecular weight around 1.8 KD. Nonetheless, a high molecular weight peptide complex also seems to play a key role as a cadmium binding protein (Carr et al. 1998; Leopold et al. 1999). This is the case of *Acetabularia acetabulum*, in which polypeptides of molecular weight higher than 100 KD seem to bind with the metal (Karez et al 1989).

The synthesis of phytochelatins, or metallothionines class III, seems to be related to the secondary metabolism of glutathione (Steffens, 1990). Therefore, titration of the acid soluble, or TNP, is currently considered the way to measure the synthesis of phytochelatins (Ric de Vos et al, 1992).

The significant increase of TNP observed in *Acetabularia calyculus* when exposed to cadmium, suggests a direct relationship between the presence of the metal in the cells and the increase of phytochelatins derived from glutathione. This relationship has also been found by researchers working on plants exposed to heavy metals (Rijistenbilet et al, 1998; Pandey et al. 1999). Our results suggest that an increment of the synthesis of peptides with low molecular weight, which bind 70% of the intracellular cadmium, is related to the acceleration of the synthesis of glutathione, which seems to act as a precursor of phytochelatins (Zenk et al 1996).

Table 1. Cadmium content (average ± SD) of total protein fractions obtained by gel filtration chromatography of extracts of exposed cells.

Fractions (pooled-2ml)	UgCd	UgCd/ml	%Cd	Total % Cd
Peak I	0.664+0.001	0.166+0.002	11.44	11.44
Peak II	0.452+0.001	1.113+0.001	7.78	14.67
	0.400+0.160	0.100+0.040	6.89	
Peak IV	0.469+0.160	0.116+0.040	7.99	73.89
	0.744+0.220	0.186+0.055	12.82	
	2.188+0.187	0.547+0.046	37.70	
	1.892+0.160	0.223+0.040	15.38	

Each value within each peak corresponds to a fraction of 2 ml. Cadmium content of the remaining fractions is not shown because it was lower than the detection limit (0.003 mg/l).

Table 2. Concentration totals (micromolar sulphydrils / grams fresh weight) of total thiol (TT), non-protein thiol (TNP) and protein thiol (TP) in extracts of soluble total protein, from control and exposed cells (to $CdCl_2$ (20mg/l)).

Thiol Groups	Control Cells µM SH/g.f.w.	Cd Exposed Cells µM SH/g.f.w.
TotalThiol (TT)	0.160± 0.034	0.215±0.062
Non-Protein Thiol (TNP)	0.026±0.003	0.111±0.035*
Protein Thiol (TP)	0.125±0.022	0.161±0.065

Values are averages ± SEM (n=6) * $p<0.05$ compared the groups.

We conclude that the capability of this algae to bioconcentrate large amounts of metal is related to: (a) the induction of a peptide of 14 KD; (b) a significant increase of the synthesis of constitutive low molecular weight peptides, which bind up to 70% of the intracellular cadmium, and exhibit a high content of sulphydryl groups; and (c) a significant increase of TNP, which is related to an increment of glutathione content in the cells. According to these results, we propose that *Acetabularia* responds to the exposure to heavy metals by synthesizing phytochelatin-like molecules.

We are currently working on elucidating the biosynthetic origin of these proteins, as well as their intracellular location.

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