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Characterization of Cd-induced low molecular weight protein in a N_2-fixing cyanobacterium *Anabaena doliolum* with special reference to co-/multiple tolerance

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This study presents information on the production of a 3.3 kDa protein, rich in cadmium and –SH contents and sensitive to buthionine-sulfoximine (BSO), in a diazotrophic cyanobacterium *Anabaena doliolum* following 20 μM Cd exposure. The absorbance at A_{254} was lost on acidification and could not be resumed even after neutralization. The radioactive sulfur ($H_2{}^{35}SO_4$) labeling depicted maximum incorporation of ^{35}S in the 3.3 KDa fraction. Synthesis of this protein was blocked by transcriptional and translational inhibitors, and resumed on glutathione supplementation. This suggests that its synthesis is independent of genetic regulation. The synthesis of this protein was stimulated by $CoCl_2$ and inhibited 85% by dark incubation and 100% by L-azaserine and DCCD. This demonstrates the participation of energy in its synthesis. Compared to the untreated control the Cd-pregrown *A. doliolum* however, showed an increased final yield and higher tolerance index (TI) when exposed to metals like copper, nickel, lead, iron and zinc, anaerobiosis, heat, and cold shocks as well as X-rays and UV-B irradiations. This study suggests that the low molecular weight cadmium-induced protein of *A. doliolum* largely resembles the higher plant phytochelatins (PCs) and offers not only co-tolerance to different heavy metals but also provides multiple tolerance to a host of environmental stresses.

Keywords: *Anabaena doliolum*, heat and cold shocks, phytochelatins, UV-B radiation, X-rays

Introduction

The production of metal binding proteins in plants is an adaptive response and distinctive in the sense that heavy metals are the primary inducers. Many researchers are of the opinion that higher plants, algae and some fungi produce different class of metal binding proteins, unlike metallothioneins which occur in animals. The presence of several γ-carboxymide linkages in this polypeptide was the turning point which classified them as class III MTs/phytochelatins/PCs (Robinson 1989). Synthesis of this protein has been found to be conserved from Orchidales, the most advanced group of higher plants to red, green and brown algae (Gekeler *et al.* 1988, Shaw *et al.* 1989, Ahner & Morel 1995, Inouhe *et al.* 1996). Purified peptides of this protein are known to be composed of three amino acids; namely L-glutamic acid, L-cysteine and glycine. However, biosynthesis of these peptides (PCs) has clearly indicated that they are not primary gene products. Their synthesis is known to be catalyzed by a specific γ-glutamyl cysteine dipeptidyl transpeptidase, called

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phytochelatin synthase, which is activated in presence of metal ions and uses glutathione as substrate for the post-translationally activated metal-dependent enzymatic pathway (De vos *et al.* 1992). Studies conducted by Robinson *et al.* (1988) demonstrate that synthetic deoxyribonucleotide sequences encoding the first metal binding site did not hybridize with mRNA from *Datura innoxia* cells growing in excess cadmium. Moreover, addition of cyclohexamide that inhibited overall protein synthesis still allowed considerable cadmium-induced PCs formation in *D. innoxia*, thereby demonstrating that PCs are the secondary metabolites. However, it is pertinent to mention that all the above studies have been conducted in eukaryotic systems.

Contrary to the above, the cadmium-induced metal binding protein of prokaryotic cyanobacterium *Synechococcus* Tx-20, resembles the class II metallothionein (MT II) and its production is regulated at transcription level (Olafson *et al.* 1988). Besides, the Cd-binding protein of *Synechococcus* PCC-6301 is also found to be a gene product and gene *Smt A* has been characterized (Morby *et al.* 1993, Turner *et al.* 1993). Morby *et al.* (1993) have also shown that gene *SmtB* is a metal dependent repressor of cyanobacterial metallothionein gene *SmtA*. These reports amply demonstrate that nitrogen-fixing cyanobacteria have been least explored in this regard.

The nitrogen-fixing cyanobacteria constitute an important group in plant kingdom being endowed with O_2-evolving photosynthesis on one hand and nitrogen fixation on the other. Our earlier study (Mallick *et al.* 1994) has demonstrated induction of a low molecular weight Cd binding protein in a N_2-fixing cyanobacterium *Anabaena doliolum*. However, this protein has not been characterized and nothing is known about its role in offering co-tolerance or multiple tolerance to other metal ions. Because of the prokaryotic nature of the test organism it was hypothesized that the Cd-induced protein of *A. doliolum* could be of MT II type, as reported for *Synechococcus* (Olafson *et al.* 1988, Morby *et al.* 1993). The main focus of this study was, therefore, (i) to measure molecular weight of the Cd-induced protein of *A. doliolum* using gel permeation chromatography and to study its synthesis in the presence of transcriptional and translational inhibitors, and (ii) to check if glutathione, BSO, DCCD, $CoCl_2$, L-azaserine and light/dark incubations can regulate its synthesis. Special emphasis has been given to study how this protein responds to other metals (co-tolerance) and environmental stresses (multiple tolerance).

Materials and methods

Test system

Anabaena doliolum Bharadwaja was grown axenically in modified Allen & Arnon's medium (1955) at pH 7.5 under 72 μmol photon $m^{-2}s^{-1}$ PAR light intensity and a photoperiod of 14: 10 h at 24 ± 2°C. Stock solution of $CdCl_2 \cdot 2.5\ H_2O$ was prepared in double glass distilled water and passed through Millipore membrane filter (0.22 μm) before use. Biochemicals used were obtained from Sigma Chemical Co. USA and Glaxo, India.

Gel permeation chromatography

A dense culture (0.4 O.D. at 663 nm) of *A. doliolum* was grown in presence of 20 μM $CdCl_2 \cdot 2.5H_2O$ for 7 days and the cells were harvested by centrifugation. Cells (40g fresh wt.) once washed in 50 mM potassium phosphate buffer (pH 8.0) were resuspended in the same buffer supplemented with 5 mM mercaptoethanol + 1 mM PMSF and disrupted by sonication. The homogenate was centrifuged for 30 min at 20 000 r.p.m. and the supernatant was heated at 60 °C for 3 min. The supernatant subjected to $(NH_4)_2SO_4$ (80%) precipitation for 3 h was collected, dialysed against the same buffer and lyophilized. The sample so obtained was subjected to DEAE cellolose column (25 ml) and 10 column volume of the above buffer was allowed to pass out. Bound materials were eluted with same buffer spiked with 0.5 M NaCl. The fractions thus collected were tested for –SH and Cd contents. The Cd and –SH rich fraction so collected was subjected to ultrafiltration (Centrisart-C30, Sartorius, Germany) followed by dialysis and lyophilization. The lyophilysed sample was diluted with Tris-HCl (0.01 M) buffer supplemented with KCl (0.1 M, pH 7.0) and chromatographed on a sephadex G-50 column (40 × 1.0 cm, flow rate: 6 ml h^{-1}). Fractions of 2.3 ml were collected and the absorbance was recorded at 254 nm. The column was, however, calibrated previously with carbonic anhydrase (Mol. wt. 29 000), Tripsin inhibitor (Mol. wt. 20 100), Lysozyme (Mol. wt. 14 400), cytochrome-c (Mol. wt. 12 400), Aprotenin (Mol. wt. 6500) and vitamine-B_{12} (Mol. wt. 1300) and standard curve was prepared.

Spectrophotometric pH titration

The fractions pooled by gel filtration were acidified (1 N HCl) and neutralized (1 N NaOH) according to Weber *et al.* (1987). Absorbance of the acidified and neutralized samples was recorded at 254 nm.

Analysis of –SH, GSH, PC–SH and radioactive sulfur

The level of total acid soluble -SH was determined according to Ellman (1959). GSH content of the cells was measured by the method of Anderson (1985). The PC-SH was quantified as the total acid soluble SH minus GSH (Knecht *et al.* 1994). For measuring the incorporation of

sulfur the test organism was grown in presence of radioactive sulfur ($H_2{}^{35}SO_4$) for 2 h and the protein samples for gel filtration were prepared in the manner described earlier. The radioactive counts of the eluted fractions were measured with the help of a liquid scintillation counter (Beckmann model LS-6500).

Analysis of co-tolerance

(a) Metals. To analyze co-tolerance of the Cd-induced phytochelatins to other metals the Cd-pregrown and untreated (control) *A. doliolum* were exposed to sublethal concentrations of Cu, Ni, Zn, Pb and Fe and final yield was recorded by measuring the absorbance at 663 nm on 15th day. Tolerance index (*TI*) was computed according to Baker (1987):

$$TI\ (\%) = \frac{\text{mean yield in treated culture}}{\text{mean yield in control culture}} \times 100$$

(b) Anaerobiosis, heat and cold shocks. To determine the ability of Cd-induced PCs to tolerate other environmental stresses, cells of Cd-pretreated and untreated *A. doliolum* were subjected to anaerobiosis (2 mM Na_2S for 24 h), heat shock (45 °C in a water bath for 2 h) and cold shock (4 °C for 24 h). Cells so treated were brought to normal growth conditions and final yield was recorded on 15th day. The tolerance index (*TI*) was calculated as described earlier.

(c) Ionizing and non-ionizing radiation. X-rays and UV-B irradiation were selected for this study. The Cd-pretreated and untreated (control) *A. doliolum* were exposed to sublethal doses of UV-B (12.9 m Wm^{-2} nm^{-1} for 25 min) and X-ray (1 Gy) and the tolerance was compared by computing the tolerance index.

Statistical analysis

The results were analyzed with the help of Student's *t* test and correlation coefficient (*r*).

Results and discussion

The elution profiles of cell-free extracts of Cd-treated and untreated (control) *A. doliolum* are presented in Figure 1. In control the absorbance at 254 nm showed a peak in fraction no. 6. However, in Cd-treated cells, besides fraction no. 6, an increased absorbance (optical density = 2.12) was noticed in fraction no. 13. As shown in the inset of Figure 1 the molecular weight of the fraction no. 13 was 3.3 KDa. The absorbance of fraction no. 13 was reduced by 98% following acidification and could not be restored even after neutralization with 1 N NaOH (insignificant 2% recovery; $P > 0.05$, Student's *t* test, Figure 1). In contrast to complete recovery of mamallian metallothionein a 96% loss in absorbance suggests that an irreversible change

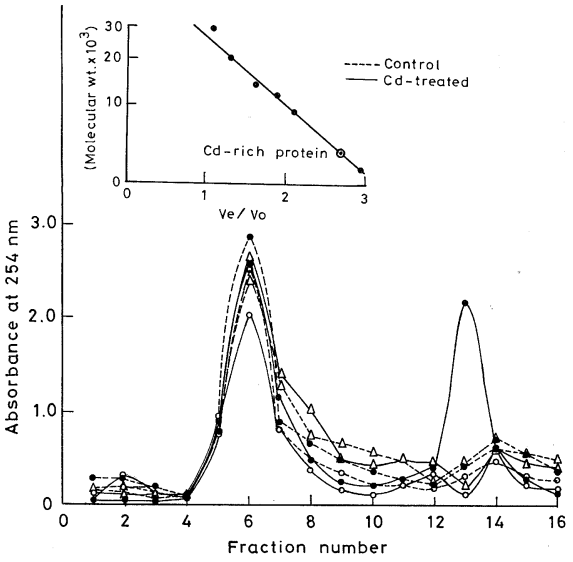

Figure 1. Elution profile of gel-permeation chromatography of Cd-treated (—) and untreated (- - -) *A. doliolum* on sephadex G-50 column. Control (●- - -●), Cd-treated (●—●), acidified control (○- - -○), acidified Cd-treated (○—○), neutralized control (△- - -△) and neutralized Cd-treated (△—△). Molecular weight of the Cd-induced protein (inset).

has occurred following acidification (Hamer, 1986). This irreversible loss could be due to the presence of acid-labile sulfur ions in the said fraction; the presence of acid labile sulfur was also confirmed by DTNB reaction (Ellman's reagent test, Figure 2) and radioactive sulfur (^{35}S)-labelling experiment (Figure 3).

For further characterization, synthesis of this protein was studied in presence of buthionine-sulfoximine (BSD, an inhibitor of glutathione synthesis), rifamycin (transcriptional inhibitor) and chloramphenicol (translational inhibitor). The test cyanobacterium produced about 11.5 nmol. PC-SH mg^{-1} protein after 7 days of Cd-treatment (Figure 4). However, an insignificant ($P > 0.05$, Students's *t* test) amount of PC-SH was detected in Cd-treated *A. doliolum* cells pretreated with BSO, rifamycin and chloramphenicol 2 h before the Cd-treatment. However, when cells pretreated with above chemicals were spiked with 10 $\mu g\ ml^{-1}$ glutathione, synthesis of PC-SH was resumed; being maximum in case of (Cd + chloramphenicol + glutathione) followed by (Cd + rifamycin + glutathione). The inhibition of synthesis of the Cd-induced protein of *A. doliolum* in presence of transcriptional and translational inhibitors, and its restoration in glutathione supplemented cultures suggests that unavailability of glutathione is responsible for blocking of synthesis of this protein. Moreover, it also confirmed that the

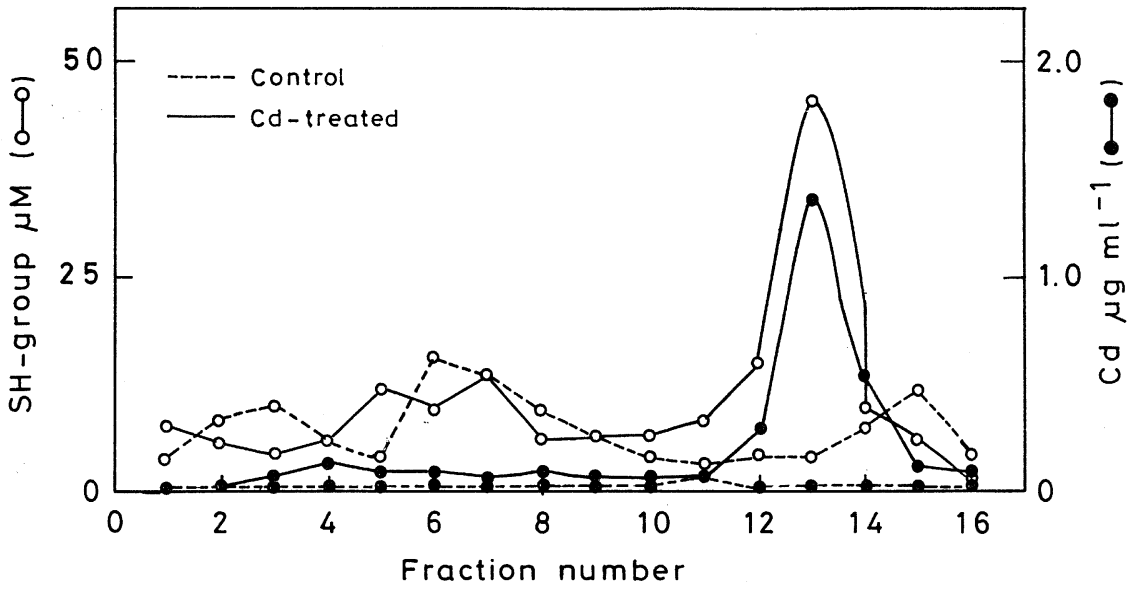

Figure 2. Cd (●) and sulfur (○) contents of the fractions eluted from sephadex-G-50 column of control and Cd-treated *A. doliolum*.

Cd-induced protein of *Anabaena doliolum* is independent of genetic regulation as known for higher plants and eucaryotic algae, and differs from *Synechococcum* where phytochelatin production is transcriptionally regulated (Olafson *et al.* 1988).

Contrary to this, synthesis of this protein was inhibited in BSO-pretreated cells (Figure 4); this inhibition could not be restored even after glutathione supplementation. The nonresumption of PCs synthesis in Cd + BSO + glutathione supplemented cells could be due to the complete blocking of glutathione resynthesis due to presence of BSO which is a known inhibitor of the enzyme γ-glutamylcysteine sythetase. A 26% increase in PC-SH of *A. doliolum* ($P < 0.05$, Student's t test) in 0.1 mM $CoCl_2$-treated cells (Figure 5), could be due to the reported increase in glutathione level of the cell supplemented with cobaltous ions (Sasame & Boyd 1978). Since glutathione is the precursor of phytochelatin, an increased glutathione concentration is expected to increase phytochelation synthesis. A highly significant positive correlation (r = 0.85,

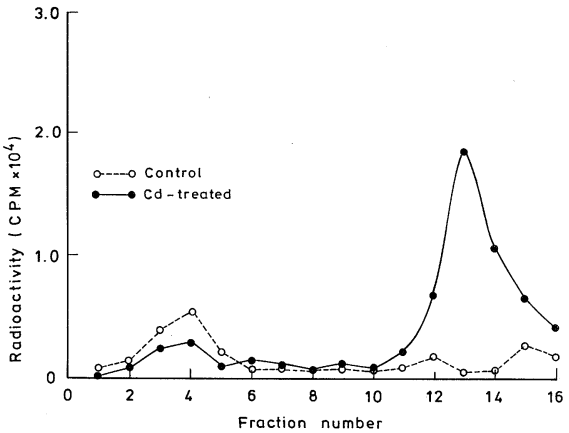

Figure 3. Elution profile of extracts of *A. doliolum* pregrown in absence and presence of $CdCl_2$ (20 μm). Cells either grown in presence or absence of $CdCl_2$ were labeled with ^{35}S for 2 h.

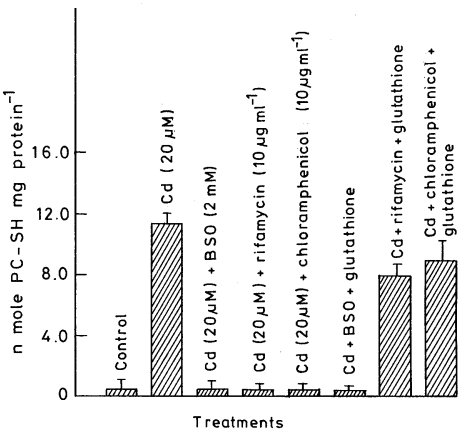

Figure 4. Synthesis of PC–SH in presence of inhibitors of transcription, translation and enzyme γ-glutamylcysteine synthetase.

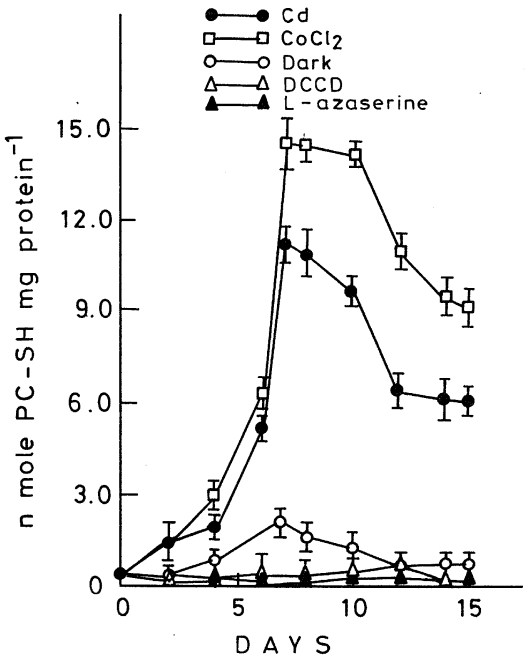

Figure 5. Factors affecting PC–SH production.

$P < 0.01$) between glutathione concentration and phytochelatin content of the cells offers testimony to the above view.

That Cd-induced low molecular weight protein of *A. doliolum* is phytochelatin was further confirmed when its synthesis was studied in presence of L-azaserine (Figure 5). A complete depression of PC synthesis in 0.01 mM L-azaserine-treated cells could be due to the inhibiton on the enzyme γ-glutamyl transpeptidase (Sasame & Boyd 1978), responsible for PC synthesis.

Figure 5 further demonstrates a complete loss of PC synthesis in presence of DCCD (0.1 mM), which inhibits ATP synthase by blocking the proton flow through F_O unit (Peschek *et al.* 1988). The inhibition of PC synthesis following DCCD supplementation demonstrates the essentiality of energy for its synthesis. A 85% ($P < 0.05$, Student's t test) reduction in PC synthesis in dark incubated cells (Figure 5), reconfirmed the role of energy in PC synthesis, as light driven primary photochemical reactions are the main source of energy in cyanobacteria (Bottomley & Stewart 1976).

Table 1. Effects of Cu, Ni, Zn, Fe and Pb on final yield and tolerance index (*TI*) of untreated and Cd-pregrown *A. doliolum*

Treatment	Final yield (O.D. at 663 nm)	
	Untreated	Cd-pregrown
Control	0.42 ± 0.11 (–)	0.35 ± 0.006 (–)
Cu (0.5 μg ml^{-1})	0.20 ± 0.017 (48)	0.23 ± 0.011 (66)
Ni (0.5 μg ml^{-1})	0.22 ± 0.010 (52)	0.25 ± 0.006 (71)
Fe (5.0 μg ml^{-1})	0.28 ± 0.006 (66)	0.51 ± 0.017 (146)
Pb (5.0 μg ml^{-1})	0.19 ± 0.011 (45)	0.24 ± 0.006 (68)
Zn (5.0 μg ml^{-1})	0.25 ± 0.006 (61)	0.49 ± 0.017 (140)

All the values are mean ± SE. Data in parentheses represent *TI* (%).
The treated groups are significantly different from control
($P < 0.01$, Student's t test).

Table 2. Impact of various stresses on final yield and tolerance index (*TI*) of untreated and Cd-pregrown *A. doliolum*

Treatment	Final yield (O.D. at 663 nm)	
	Untreated	Cd-pregrown
Control	0.43 ± 0.017 (–)	0.34 ± 0.011 (–)
Heat shock	0.04 ± 0.006 (09)	0.15 ± 0.011 (44)
Cold shock	0.28 ± 0.015 (65)	0.26 ± 0.006 (76)
Anaerobiosis	0.15 ± 0.011 (35)	0.21 ± 0.011 (62)
X-rays	0.16 ± 0.011 (37)	0.23 ± 0.017 (68)
UV-B	0.21 ± 0.006 (49)	0.20 ± 0.011 (59)

All the values are mean ± SE. Data in parentheses represent *TI* (%).
The treated groups are significantly different from control
($P < 0.01$, Student's t test).

Effect of sublethal concentrations of Cu, Ni, Zn, Pb and Fe on final yield and tolerance index (*TI*) of untreated control and Cd-pregrown *A. doliolum* is given in Table 1. The tolerance index (*TI*) of Cd-pregrown cells was significantly ($P < 0.01$, Student's *t* test) high as compared to the untreated control i.e. 18, 19, 80, 23 and 79% higher respectively for Cu, Ni, Fe, Pb and Zn. Increased tolerance index could be due to detoxification of metals through binding with sulfur ions and Cd-induced phytochelatins, thus demonstrating co-tolerance in the Cd-pregrown cells.

Table 2 demonstrates the tolerance of Cd-pregrown cells against different environmental stresses. Cadmium pregrown cells exhibited significant increase in tolerance index (*TI*) against heat shock (35%), X-rays (31%), anaerobiosis (27%), and less significant to cold shock (11%) and UV–B (10%) radiation. Cross tolerance between heavy metals and heat stress has been reported by Bonham-Smith *et al.* (1987) and Orzech & Burke (1988). Huang & Goldsbrough (1988) also found that the Cd tolerant tobacco cell cultures showed more tolerance to both heat and cold treatments than its untreated control. This non-specific tolerance ability of metal-induced phytochelatins has also been reported by Baker (1987). Thus, the multiple tolerance could be due to production of some more general stress proteins in Cd-pregrown cells, which not only offer tolerance to Cd and other metal ions but also to a variety of stresses.

This study therefore, clearly demonstrates that production of this protein is not genetically regulated in *A. doliolum*. This protein not only enables the cyanobacterium to co-tolerate other metals but also provides multiple tolerance to a variety of environmental stresses.

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