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Photosynthetic Protein from *Chlorella vulgaris* Strain Bt-09 May Be Responsible for the Coping Mechanism Against Cadmium Toxicity

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Chlorella vulgaris has been observed to bioaccumulate significant amounts of Cd ions from the medium (Carr *et al.*1998; Matsunaga *et al.* 1999). However, the actual biochemical mechanism responsible for its tolerance to Cd has not been fully elucidated. Widely reported coping mechanisms for various microalgae include exclusion, binding to cell wall substances, and binding to cytosolic proteins. Whether or not *C. vulgaris* utilizes any of these mechanisms is not yet established. Metal ions, once inside the cell, may bind to cytosolic components such as high affinity specific ligands, substrates, products of enzymatic activity, or enzymes themselves (Venkataraman *et al.* 1992). These binding components may act as chelators or carriers. In photosynthetic organisms, the general classes of known metal chelators included phytochelatins, metallothioneins (Grill *et al.* 1987; Gekeler *et al.* 1989; Rauser 1995), organic acids, and amino acids (Clemens 2001). Most cell components are susceptible to metal-induced damage as these contain metal-sensitive enzymes (Rouch *et al.* 1995). One of these components is the chloroplast which is vital to the photosynthetic activity of the organism. This study reports the mechanism by which *C. vulgaris* strain Bt-09 cells tolerant to 0.5 mg/L $CdCl_2$ cope with Cd stress.

MATERIALS AND METHODS

Pure cultures of *C. vulgaris* strain Bt-09 cultures were grown aseptically in BG-11 liquid medium at 27 ± 2 ^{0}C under constant illumination (60 $\mu Em^{-2}s^{-1}$). Cultures designated for Cd treatment were grown for 18 days in Cd free media, after which Cd ($CdCl_2$, Titrisol, 1,000 ppm Cd-Std) solution was added to give a final concentration of 0.5 mg/L and reincubated for 2 more days prior to harvest. Cd-treated cultures were harvested by high speed centrifugation (10,000 x *g*, 10 min). The recovered whole cell pellet was washed with ultrapure water, resuspended in 0.1 *M* Tris-HCl (pH 8.0) buffer and sonicated. The disrupted cells were centrifuged at 1,000 x *g*, 10,000 x *g*, and 100,000 x *g* for 10, 20 and 120 min, respectively. The post-microsomal fraction was derived from the supernatant collected after the 100,000 x *g* centrifugation. An aliquot of this supernatant was run in SDS-PAGE, while the rest was flash frozen in liquid nitrogen and was stored at -20 ^{o}C prior to analyses. This post-microsomal fraction was subjected to size fractionation using 2 types of dialysis tubings. Initially, the supernatant was transferred into NMWL < 12,000 Da dialysis tubings, each tubing placed into a

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14-mL tube, spun (3,000 x *g*, 6-8 hr), and the subsequent retentate and filtrate were recovered. The retentate was subjected to AAS analysis (1×10^{-4} ppm), while the filtrate was transferred into NMWL <2,000 Da dialysis tubings, sealed and covered with dry PEG-4000. An aliquot of this concentrated solution was run in SDS-PAGE while the remaining fraction was subjected to AAS analysis.

Size exclusion chromatography was employed in the attempt to isolate Cd-binding proteins. The cytosolic fraction was initially decolorized by multiple passes through a dry Sephadex G-10 mini-column. The 3-mL decolorized cytosol was then loaded onto a Sephadex G-50 column, equilibrated with 10 m*M* NH_4HCO_3 solution containing 200 m*M* KCl. Fractions were collected at a flow rate of 3 mL/min and absorbance of each eluted fraction was determined at 254 and 280 nm.

The post-microsomal supernatant was fractionated using anion-exchange chromatography. Five volumes of samples were thoroughly mixed with 5 volumes of 50 *mM* Tris-HCl-equilibrated QAE [2-hydroxypropyl] aminoethyl Sephadex A-25 (pH 8.0). The mixture was allowed to stand for an hour, centrifuged (10,000 x *g* , 4°C, 10 min) and the supernatant designated as F_1. The recovered QAE matrix was washed with room temp Tris-buffer. A series of subsequent resuspensions and centrifugations of the QAE in 0.06, 0.1, 0.25, 0.4, and 1 *M* KCl-Tris-HCl solutions were accomplished and these collected supernatants were each concentrated by dialysis tubings-PEG system. The concentrated collected fraction was subjected to SDS-PAGE and AAS for the detection of Cd, Cu and Zn.

The F_1 fraction was further concentrated, run on SDS-PAGE gels, electroblotted onto PVDF membrane (250 V, 2 ½ hr) in Tris-Glycine buffer (25 m*M* tris-base pH 8.3, 192 m*M* glycine, 15% methanol). The portion containing the 3 kDa protein band was excised and analyzed at the HHMI Biopolymer/ W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, USA for the amino acid composition and sequence.

The secondary structure of the polypeptide was predicted using the Chou-Fasman secondary structure protein prediction program (http://fasta.bioch.virginia.edu/o_fasta/cgi/garnier.cgi). All procedures were conducted in three replications except for the amino acid analyses.

RESULTS AND DISCUSSION

The protein profiles of the post-microsomal fraction of *C. vulgaris* strain Bt-09 showed the presence of novel proteins, M_r's of 23 and 3 kDa (Figure 1), presumably induced by exposure to Cd. Our separation scheme using two dialysis tubings with different NMWLs, followed by AAS analysis, unequivocally showed that the low M_r polypeptide was indeed associated with Cd ions (Figure 2).

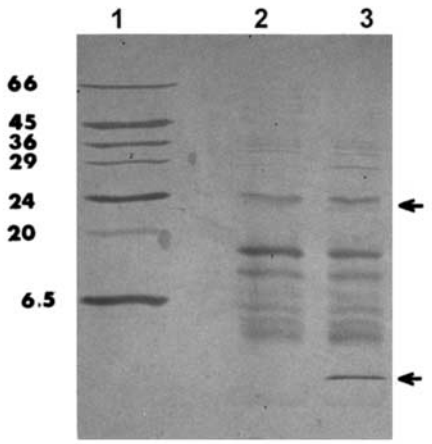


Figure 1. 15% SDS-PAGE of *C. vulgaris* strain Bt-09 post- microsomal fraction exposed to 0.5 mg/L $CdCl_2$ for 2 days. The molecular markers were loaded in lane 1. Cytosolic extracts from the untreated and treated cultures of *C. vulgaris* cells were loaded in lanes 2 and 3, respectively. The arrowheads correspond to 23 and 3 kDa polypeptides induced by Cd.

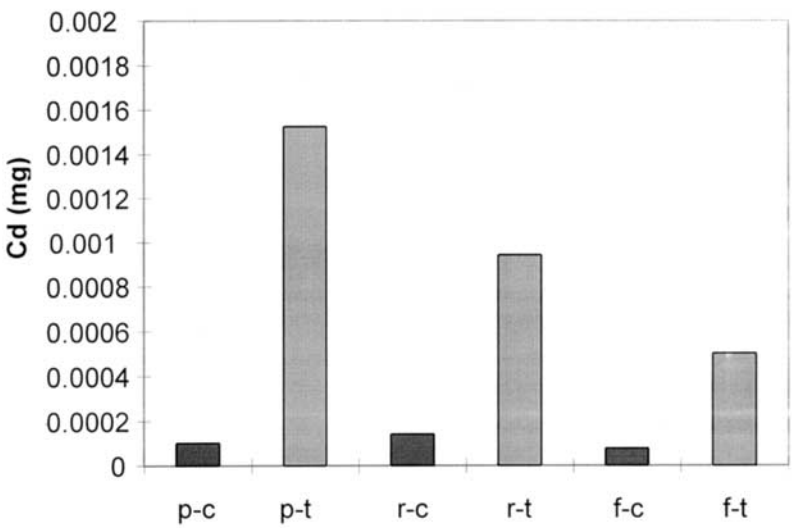
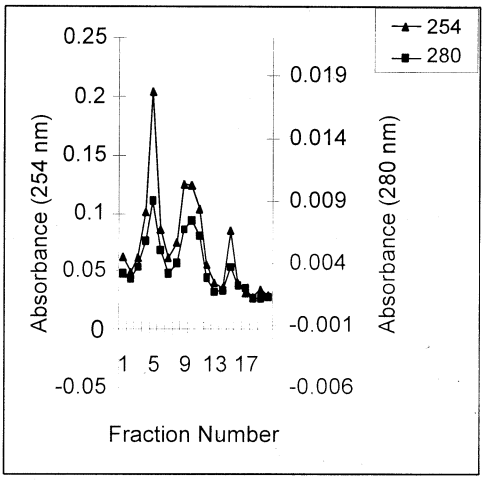


Figure 2. Cadmium content of post-microsomal supernatant (p), retentate (r) and filtrate (f) of extracts from *C. vulgaris* strain Bt- 09 exposed to 0.5 mg/L $CdCl_2$ for 2 days compared with those from the control (c) cultures.

Size exclusion chromatography of the post-microsomal fraction, as monitored by absorbance of eluted fractions at 254 and 280 nm, established the elution profile of cytosolic proteins in Cd-exposed and unexposed cultures (Figures 3A and 3B). The absorbance at 254 nm of some of the eluted fractions indicated the presence of cysteine-metal complexes. The metal component of these complexes could be any of the divalent metal such as Cd, Cu, and Zn (Everard and Swain 1983). The cysteine residue in these proteins is frequently involved in linkages with heavy metals and is likely to carry out the sequestration of Cd via the sulfhydryl functional group.

The elution profile of cytosolic proteins from the Cd-exposed cultures indicated the induction of low M_r proteins, eluting out as fractions 17-18 in Figure 3B. Presumably, one of these low M_r proteins is the 3 kDa polypeptide detected in the SDS-PAGE profile. The sequence of this 3 kDa polypeptide is shown in Figure 4. To account for the strong absorbance of fractions 17-18 at 254 nm, one must assume that at least one of the four unidentified amino acid residues is a cysteine. The Cd ions can be attached to one or more of the putative cysteine residues in the 3 kDa protein. The binding of the Cd ions to the putative cysteine residues can be effected by electrostatic attraction and/or coordinate covalent linkages.

A.



B.

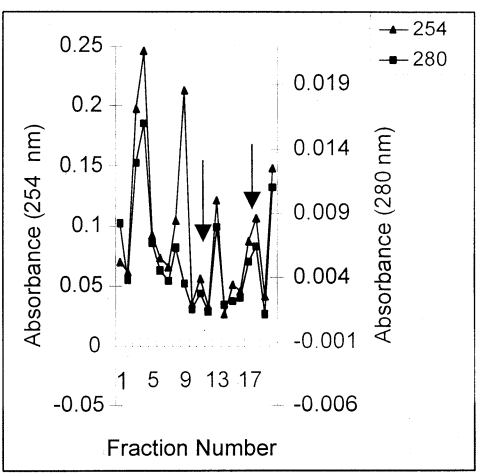


Figure 3. Size exclusion elution profile of post-microsomal fractions of untreated (A) and treated (B) cultures of *C. vulgaris* strain Bt-09 exposed to 0.5 mg/L $CdCl_2$ for 2 days. Arrows indicate fractions 11 and 17-18, presumably containing the 23 and 3 kDa proteins, respectively.

Sequence:	X	Leu	Thr	Ala	Asp	Leu	Leu	Ala	Lys
Cycle #:	1	2	3	4	5	6	7	8	9
Sequence:	Thr	Ala	Glu	Asn	Lys	Ala	Leu	Asn	X
Cycle #:	10	11	12	13	14	15	16	17	18
Sequence:	Lys	Lys	X	Leu	Ala	Ser	Ser	Tyr	X
Cycle #:	19	20	21	22	23	24	25	26	27
Sequence:	Asn	Phe							
Cycle #:	28	29							

Figure 4. Amino acid composition and sequence of the 3 kDa polypeptide induced by Cd.

Alternatively, the Cd ions could be bound to the protein by electrostatic attraction even without the coordination of the hypothetical cysteine residue(s). The possible binding sites of Cd ions in the 3 kDa protein are the negatively charged aspartate at position 5 (D5) and glutamate at position 12 (E12). Using Chou-Fasman rules for secondary structure prediction, D5 and E12 are part of the α-helical segment of the 3 kDa polypeptide (Figure 5), which extends from the leucines at positions 2 and 22. D5 and E12 are separated by seven amino acid residues. Assuming the ideal α-helical arrangement of 3.6 residues per turn, the two amino acids are expected to be on the same side of the α-helix, about two turns apart. The negatively charged carboxyl groups, which extend outward from the helix are the possible sites for electrostatic interactions with Cd ions.

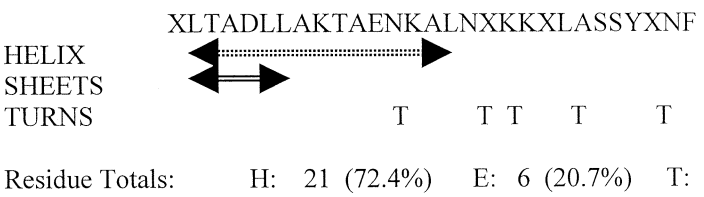


Figure 5. Predicted secondary structure of the novel 3 kDa protein of *C. vulgaris* strain Bt-09 exposed to 0.5 mg/L $CdCl_2$.

Because we were not able to isolate the 23 kDa protein in solution form, its Cd-binding capacity cannot be determined. Other proteins in the mixture may also bind Cd, and thus, the Cd associated with high M_r fraction (retained by the 12 NMWL dialysis bag) may represent the total Cd bound by several proteins found in this fraction. However, the two cysteine residues in the 23 kDa polypeptide (Table 1) present possible binding sites for Cd, possibly explaining the absorption at 254 nm of the midrange M_r protein fraction 11 (Figure 3B).

Table 1. Amino acid composition of the 23 kDa polypeptide induced by Cd.

Amino Acid	23 kDa	
	Mole Percent	# residues
Phenylalanine (Phe)	5.0	9.9
Cysteine (Cys)	0.9	1.7
Serine (Ser)	5.3	10.5
Methionine (Met)	1.4	2.8
Asparagine / Aspartate (Asx)	7.7	15.1
Glutamine / Glutamate (Glx)	12.8	25.2
Threonine (Thr)	3.6	7.0
Glycine (Gly)**	17.3	34.2
Alanine (Ala)	11.0	21.7
Valine (Val)	6.1	12.0
Leucine (Leu)	10.7	21.1
Isoleucine (Ile)	3.8	7.6
Proline (Pro)	7.6	14.9
Lysine (Lys)	2.2	4.3
Arginine (Arg)	3.7	7.3
Histidine (His)	0.9	1.8

**obscured by glycine in gel running buffer
Limited resources/ funds prevented this study to pursue amino acid sequence analysis for the 23 kDa novel protein

The 3 kDa polypeptide showed a 72% sequence similarity with the PS I fragment of *Chlamydomonas reinhardtii* (Fasta, http://www.genome.ed.jp). Binding of Cd ions to photosynthetic proteins in the chloroplast may adversely affect photosynthesis, but this adverse effect need not totally incapacitate the cells. Many chloroplasts are present in a microalgal cell and there are a number of photosystem I complexes found in the thylakoid membranes of each chloroplast.

The results of our study indicate a novel mechanism for coping with heavy metal stress. We report the first instance wherein a heavy metal was found associated with an important component in the photosynthetic apparatus, and yet the affected cells manifested no deleterious effect discernible in the growth curve and in chl ***a*** levels derived from our preliminary study. The association of Cd ions with an important component of PS I can possibly represent a heretofore unreported mechanism that the *C. vulgaris* strain Bt-09 cells use to cope with heavy metal stress. The organism need not resort to the *de novo* biosynthesis of novel stress proteins, but simply utilizes existing multicopy proteins to bind (and possibly sequester) the offending metal ions.

This mechanism may not be observed under natural conditions. Our culture of *C. vulgaris* strain Bt-09 cells was maintained under a supra-optimal condition. When cultures are grown with virtually unlimited light and inorganic nutrients, as was the case for this study, the cells may be expected to cope well with the adverse effects of Cd on their photosynthetic system. In situations where light and nutrients are limiting, the use of PS proteins for binding metals may not be a viable mechanism for coping with heavy metal stress.

The primary sequence of the 3 kDa polypeptide is very similar to the PS I sub-unit reaction center of *Chlamydomonas reinhardtii* (Fasta, http://www.genome.ed.jp). The sequence coincides particularly well with a part of Psa N protein of photosystem reaction center I. Psa N sub-unit has an M_r of about 9 kDa, which is much higher than that of our isolated 3 kDa polypeptide. We find it reasonable to assume that the deleterious effects of Cd could have fragmented the PS I Psa N sub-unit of *C. vulgaris* strain Bt-09. The dissociation and partial proteolytic fragmentation of this sub-unit from the PS I reaction center may be due to the disintegration of the thylakoid membrane as a consequence of cadmium toxicity. Since Psa N is the only extrinsic protein on the lumenal side of the thylakoid membrane (Chitnis 2001), it can easily dissociate from the membrane.

Fragmentation of chloroplasts due to Al, Fe, Cu, and Zn has been observed by Wong *et al.* (1994) on *C. fusca*. They observed that at high metal concentrations, chloroplasts were the only organelles altered. Within the chloroplast, there were disruptions of the thylakoidal membranes and enlargement of interthylakoidal spaces. We did not look at the effects of cadmium on the fine structures of *C. vulgaris* strain Bt-09. The possible use of transmission electron microscopy would be useful in verifying the possible disruption of chloroplast as affected by Cd ions.

The 3 kDa polypeptide also bound to Cu and Zn (Table 2). These ions probably competed with Cd for the putative cysteines and/or D5 and E12 residues in the 3 kDa protein. Whether or not these metals would similarly induce the appearance of the 3 kDa protein was not established by our study.

Table 2. Metal content of the 3 kDa Cd-induced polypeptide as determined by GF-AAS.

Heavy Metal	Unexposed (mg/L)	Exposed (mg/L)
Cd	2×10^{-9}	7.6×10^{-8}
Cu	1.36×10^{-6}	8.13×10^{-7}
Zn	7.223×10^{-6}	2.793×10^{-6}

C. vulgaris strain Bt-09 cells use a pre-existing PS I protein to rapidly associate with Cd or any divalent metals. The association of Cd ions with Psa N in *C. vulgaris* strain Bt-09 is apparently a way of coping with heavy metal stress. Should further experiments verify this association, our report marks the first

instance where an important component of the photosynthetic apparatus was shown to bind Cd ions.

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