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Intracellular cadmium sequestration by the heavy metal-tolerant green algae *Chlorella vulgaris* and *Uronema confervicolum*

Adam T. Wilczok, Makoto M. Watanabe[1], Sanae Kawahara, Kazuo T. Suzuki[2] and Kioshi Sugahara

National Institute for Environmental Studies, Onogawa 16-2, Tsukuba, Ibaraki, 305 Japan

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Chlorella vulgaris and *Uronema confervicolum* isolated from a metal polluted river were examined for induction of metal-binding peptide formation by exposing to 20 μM of cadmium under laboratory conditions. After three weeks of cultivation 608 mg kg^{-1} and 597 mg kg^{-1} of Cd were found in dried cells of *C. vulgaris* and *U. confervicolum*, respectively, when analyzed by the atomic absorption method. About 50% of intracellular Cd in both species was associated with the 170000 *g* cell supernatant. Distributions of cadmium in the cell-soluble fractions were determined by high-performance liquid chromatography (HPLC) with detecting by atomic absorption (AAS) or inductively coupled argon plasma-atomic emission spectrometry (ICP). Significant changes in HPLC-ICP profiles of sulfur and metals in algal cytosolic fractions were induced by the exposure to cadmium. Only one metal-binding peak was observed in *U. confervicolum*, while *C. vulgaris* induced formation of three cadmium-binding peaks on a gel filtration column. High sulfur content, heat stability and high 254 : 280 asbsorbance ratio of the induced peaks suggest similarity of the isolated Cd-binding compounds to metallothioneins found in other algae and higher plants.

Key Index Words: Cd-binding compounds—Cd-tolerance—Chlorella vulgaris—Uronema confervicolum.

When exposed to heavy metals many organisms can synthesize metallothioneins (MTs)—proteins, which play a key role in metal detoxification as well as in metal ions homeostasis (Reddy and Prasad 1990, Robinson 1989). Metallothioneins are low molecular weight heat-stable proteins characterized by high contents of heavy metals and cysteine, absence of aromatic amino acids, high 254 : 280 absorbance ratio typical for thiolate complexes, and high affinity toward anion exchangers (Kagi and Kojima 1987).

Metal-binding proteins or peptides are present or inducible in various kinds of nonmammalian species (Hamer 1986) and plants (Grill *et al.* 1987, Rauser 1990). In plants they are no primary gene products and are synthesized enzymatically from glutathione by the specific enzyme γ-glutamylcysteine dipeptydyl transpeptidase (Grill *et al.* 1989). Algal metallothioneins, most often called phytochelatins, are defined as class III MTs: nontranslationally synthesized metal-thiolate polypeptides (Fowler *et al.* 1987). Metallothionein-like metal-binding proteins, phytochelatins or other less precisely defined proteins/peptides have been found in different algae: *Anacystis nidulans, Bumilleriopsis filiformis, Chlamydomonas reinhardtii, Chlorella ellipsoidea, Chlorella fusca, Chlorella pyrenoidosa, Dunaliella bioculata, Euglena gracilis, Fragilaria crotonensis, Monoraphidium minutum, Navicula pelliculosa, Phaeodactylum tricornutum, Porphyridium cruentum, Sargassum muticum, Scenedesmus quadricauda, Stichococcus bacillaris*, and *Synechococcus* sp. (Gekeler *et al.* 1988, Hart and Bertram 1980, Heuillet *et al.* 1988, Howe and Merchant 1992, Kawaguchi and Maita 1990, Nagano *et al.* 1984, Olafson *et al.* 1980, Reddy

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[1] Address for reprint requests.

[2] Present address: Faculty of Pharmaceutical Sciences, Chiba University, Yayoi, Chiba, 263 Japan.

and Prasad 1989, Weber *et al.* 1987). Thus, the ability to synthesize metal-binding proteins or peptides seems common in the whole division of algae. While these compounds may be functionally analogous to animal MTs, their structure and biosynthesis are fundamentally different. Metal-binding compounds isolated from algae are supposed to be of identical structure to phytochelatins isolated from higher plants and described as (γ-Glu-Cys)$_n$-Gly (n=2 to 11) (Gekeler *et al.* 1988). Amino acid composition of *C. ellipsoidea* MTs consists of mainly glutamic acid or glutamine, arginine, glycine, and half-cysteine (Nagano *et al.* 1984), while in the other algae only glutamic acid, cysteine and glycine were found (Gekeler *et al.* 1988, Maita and Kawaguchi 1989). Molecular weight of different algal metal-binding proteins (or peptides) determined by gel filtration or SDS-electrophoresis is in the range of 1.8-20 kDa and markedly depends on the ionic strength applied as well as on the species tested (Grill *et al.* 1987, Hart and Bertram 1980, Lue-Kim and Rauser 1986, Murasugi *et al.* 1981, Nagano *et al.* 1984, Olafson *et al.* 1980).

Algal tolerance to heavy metals is correlated with the metal concentration in the environment where the algae were isolated. The isolates of Bacillariophyceae, Chlorophyceae, and Charophyceae from metal-polluted sites are mostly tolerant to the pollutant metal and retain their tolerance even for 2 years of subculture in the normal cultivation medium (Takamura *et al.* 1989, 1990). In particular, the chlorophycean algae, *C. vulgaris* and *U. confervicolum* can grow in high concentrations on Zn, Cu, and Cd. When tested for photosynthetic activity decrease, the concentrations of Cd equal to 25.0 mg l^{-1} for *C. vulgaris* and 16.6 mg l^{-1} for *U. confervicolum* caused 50% inhibition of photosynthesis (Takamura *et al.* 1989).

Recently, simultaneous determination of multielements including heavy metals and sulfur in different biological samples by HPLC-AAS and HPLC-ICP was proven as the useful tool in metal-binding proteins investigation (Sunaga *et al.* 1987, Suzuki 1991, Suzuki *et al.* 1987, 1988). In the present study, as a first step in our studies on characterization of metallothionein-like metal-binding compounds induced in *C. vulgaris* and *U. confervicolum*, we tried to determine distribution profiles of Cd and other elements in the supernatant derived from the algae by using both the HPLC-AAS and HPLC-ICP methods.

Materials and Methods

Unialgal cultures of *Chlorella vulgaris* Beij. (strain NIES PS-511) and *Uronema confervicolum* Lagerh. (NIES PS-526) were obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES). The strains were originally isolated from heavy metal polluted Miyata river in 1987 and deposited at the NIES-Collection (Takamura *et al.* 1989, Watanabe and Satake 1991).

Cells were cultured axenically for three weeks in the "C" medium composed of $Ca(NO_3)_2 \cdot 4H_2O$-150 mg l^{-1}, KNO_3-100 mg l^{-1}, β-Na_2-glycerophosphate-50 mg l^{-1}, $MgSO_4 \cdot 7H_2O$-40 mg l^{-1}, vitamin B_{12}-0.1 μg l^{-1}, biotin-0.1 μg l^{-1}, thiamine·HCl-10 μg l^{-1}, $FeCl_3$-588 μg l^{-1}, $MnCl_2 \cdot 4H_2O$-108 μg l^{-1}, $ZnSO_4 \cdot 7H_2O$-66 μg l^{-1}, $CoCl_2 \cdot 6H_2O$-12 μg l^{-1}, $Na_2MoO_4 \cdot 2H_2O$-7.5 μg l^{-1}, Na_2 EDTA$\cdot 2H_2O$-3 mg l^{-1}, and tris (hydroxymethyl) aminomethane (Tris)-500 mg l^{-1} (pH 7.5) (Watanabe and Satake 1991) in the foam stopped 2 l Erlenmeyer flasks under illumination of ca. 100 μmol photon m^{-2} s^{-1} with a photoperiod of 12 h light: 12 h dark from the daylight fluorescent tubes at 20°C. For Cd-treatment $CdCl_2$ was added at a concentration of 20 μM at the beginning of each experiment.

Algae were harvested by filtration through a 1.0 μm Nucleopore filter under reduced pressure, washed with 0.1 M Tris-HCl buffer (pH 7.4) and homogenized in 10 ml of the same buffer using a VR 200 P homogenizer (Tomy-Seiko, Tokyo) in an atmosphere of nitrogen gas under ice-water cooling. Dry weight was determined by drying samples to a constant weight as recommended by Sorokin

(Sorokin 1973). Three 0.5 ml aliquots of each homogenate were wet-digested with 0.5 ml of mixed acids ($HNO_3 : HClO_4$, 5 : 1). The remaining portions of homogenates were diluted with Tris-HCl buffer (0.1 M, pH 7.4) to the dry mass concentration 20 mg ml^{-1}. The 7 ml aliquots were centrifuged at 170000 *g* for 60 min at 2°C. An atomic absorption spectrometer equipped with graphite furnace (Shimadzu AA 640-12) was used to measure metal concentration in the cultivation medium, digested homogenate, and crude supernatant.

The separation of algal Cd-binding compounds was performed on two kinds of columns and elution conditions; the GS column (a gel filtration column with low interactions between column coating and substrates by elution at neutral buffer conditions) and the SW column (a gel filtration column with stronger interactions of metals between column material and substrates by elution at slightly basic pH, which better separates rat metallothioneins into isoforms) (Suzuki *et al.* 1980). Aliquots (0.2 ml) of the 170000 *g* supernatant were applied on an Asahipak GS-320 column (7.6 × 500 mm; Asahi Chemical Industry, Kawasaki, Japan) and an SW column (TSK gel G3000SW, 7.5 × 600 mm with a guard column of 7.5 × 75 mm; Tosoh Co. Ltd., Tokyo, Japan). A Tris-HCl buffer solution (10 mM, pH 8.0 containing 0.1% NaN_3) was used as the mobile phase for the SW column, while 0.9% NaCl solution containing 0.05% NaN_3 was used for the GS column. The mobile phases were degassed with a Shodex Degas degasser (Showa Denko Co., Tokyo, Japan). The flow rate was maintained at 1.0 ml min^{-1} by a Gasukuro Kogyo HPLC Model 576 (Gasukuro Kogyo Inc., Tokyo, Japan). The eluate absorbances at 254 and 280 nm were measured with a programmable Spectra 200 detector (Spectraphysics) and the eluate was subsequently introduced directly into an atomic absorption spectrometer with an acetylene flame (Hitachi 170-50 A) or into a nebulizer tube of a Daini Seikosha 2500 ICP spectrometer (Seiko Instruments and Electronics Ltd., Tokyo, Japan). All the concentrations of elements were determined simultaneously according to the method described elsewhere (Sunaga *et al.* 1987, Suzuki *et al.* 1988, Suzuki 1991). The stored data were processed and converted into distribution profiles using a self-developed software and a personal computer (PC 9801, NEC, Tokyo) and XY-plotter (FP 5301R, Graphtec, Tokyo). The SW column was precalibrated with the previously described Cd-exposed rat liver supernatant (Suzuki *et al.* 1987) and aprotinin, cytochrome c, carbonic anhydrase, and albumin—gel filtration molecular weight markers (Sigma, St. Louis, USA). To determine a heat-stability of the isolated metal-binding compounds, the cell supernatants were heat-treated (70°C, 10 min) under nitrogen gas, centrifuged (5000 *g*, 10 min) and analyzed by the HPLC-ICP on the SW column as described above.

Results and Discussion

Cadmium added into the cultivation medium was easily incorporated into the algal cells. After three weeks of cultivation 608 mg kg^{-1} and 597 mg kg^{-1} of Cd were found in dried cells of *C. vulgaris* and *U. confervicolum*, when analyzed by the atomic absorption method. 49.5% of intracellular Cd in *C. vulgaris* and 51.4% in *U. confervicolum* was associated with the 170000 *g* cell supernatant subjected for HPLC separation. Distribution of metal bound to cytosolic fraction was determined by HPLC-AAS and for more detailed characterization by HPLC-ICP. The elution profiles of Cd and absorbance recorded at 254 and 280 nm during separation of Cd-exposed *C. vulgaris* and *U. confervicolum* supernatants on the GS column are presented in Fig. 1. Both analyzed strains synthesized Cd-binding compounds. Cd-peak followed by the high absorbance at 254 nm was eluted at a retention time of 10.5 min on a GS-320 column in both species. The Cd-distribution profile in the supernatant obtained from Cd-treated *C. vulgaris* suggests the presence of isoforms or three successive metal-binding components of reten-

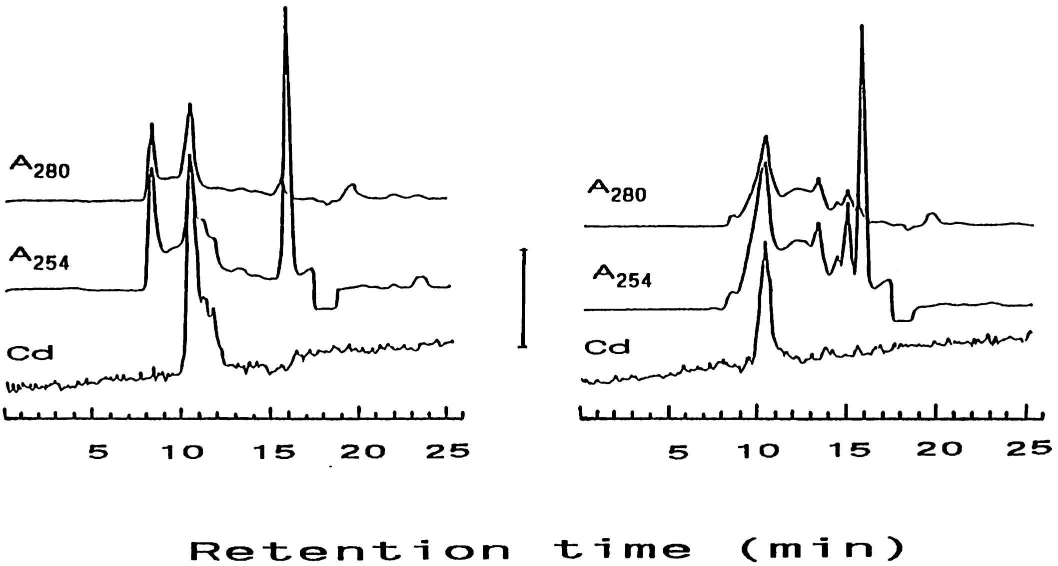

Fig. 1. Elution profiles of supernatants from Cd-exposed *C. vulgaris* (left) and *U. confervicolum* (right) on an Asahipak GS-320 column. Absorbances at 254 and 280 nm were recorded in the time course of analysis of metal-binding compounds. The vertical bar indicates the detector level (0.1 μg Cd ml^{-1}) by AAS.

tion times 10.5, 11.4, and 11.9 min, though two latter peaks were not well separated. This phenomenon was not observed in *U. confervicolum*, which bound Cd to the single peak only.

Figure 2 shows elution profiles of Cd and absorbance recorded at 254 and 280 nm during separation of Cd-treated *C. vulgaris* and *U. confervicolum* supernatants on the SW column. Three Cd-peaks were observed in *C. vulgaris*, while again only a single Cd-peak was found in *U. confervicolum*. From these results, the SW column was found more suitable for separation of cadmium-binding compounds and therefore chosen for HPLC-ICP measurements and heat-treatment experiments.

Figure 3 shows HPLC-ICP results obtained for Cd-treated and control *C. vulgaris* supernatants separated on the SW column.

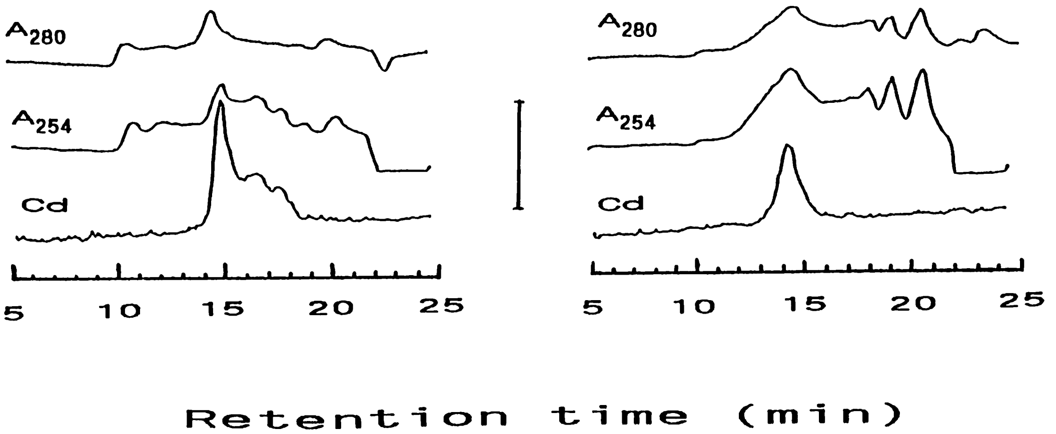

Fig. 2. Elution profiles of Cd-exposed *C. vulgaris* (left) and *U. confervicolum* (right) on a G3000SW column. The detector level (0.1 μg Cd ml^{-1}) by AAS is shown by the vertical bar.

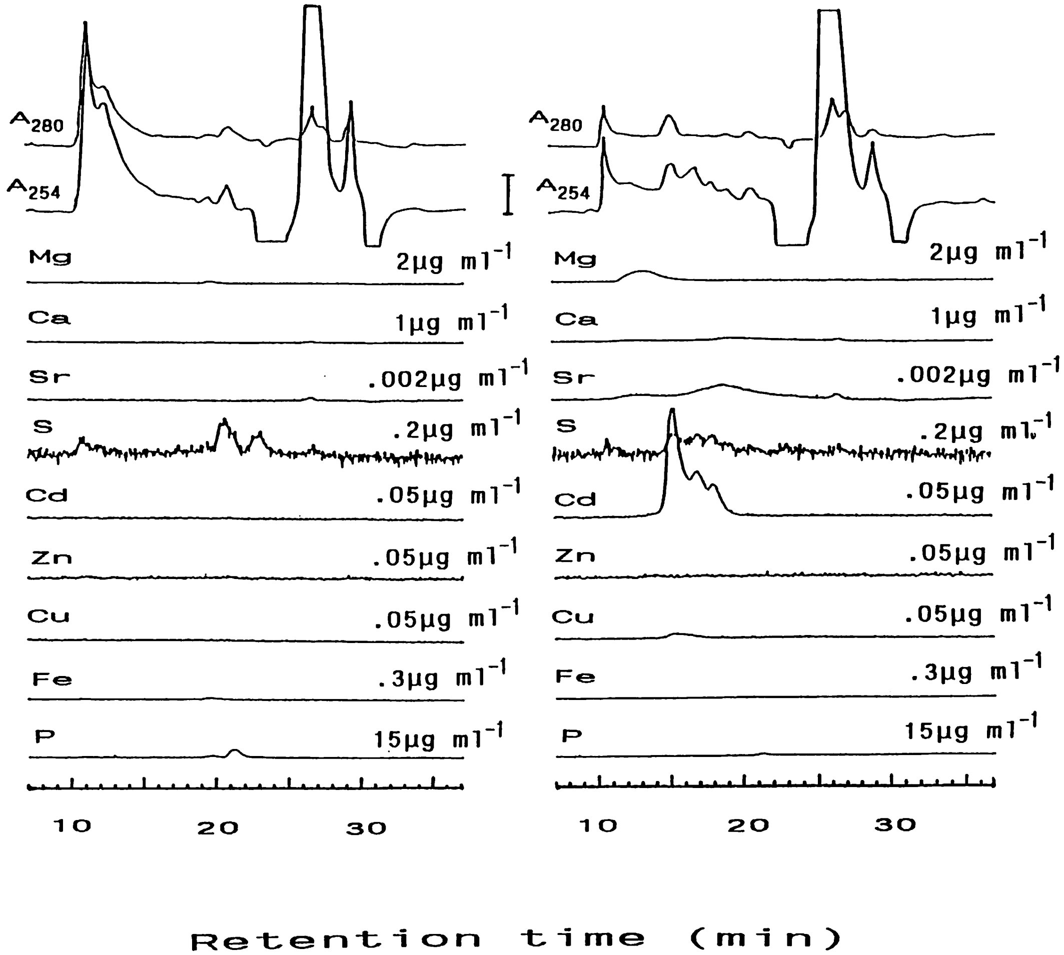

Fig. 3. HPLC-ICP profiles on a G3000SW column for the supernatants of *C. vulgaris*. Cells grown in the absence of Cd (left), cells exposed to 20 μM of Cd (right) for 3 weeks. Absorbances at 254 and 280 nm recorded in arbitrary units. The vertical bar corresponds to the detector levels of the respective elements (eg., for Cd the detector level is 0.05 μg ml^{-1}).

Metal-binding components were again eluted as three successive fractions of retention times 15.2, 16.9, and 17.8 min respectively. The low amounts of Cu found in the Cd-binding components in both algae (see also Fig. 4) suggest that the induced compounds could bind and concentrate Cu despite the very low concentration of Cu in the medium. It must be noticed, that the cultivation medium used in the present experiment did not contain Cu added as a microelement and its concentration was below the detection limit by AAS. Nagano *et al.* (1984) observed that Cu co-eluted with Cd-binding peptides, when the algae were supplied with both metals. On the other hand Zn, known phytochelatin formation inducer in *Chlorella* and *Scenedesmus* (Gekeler *et al.* 1988), which was present as a trace element in the cultivation medium, was not co-eluted with Cd-binding fractions. Probably, the higher Zn concentration is required or some antagonisms exist between Cd and Zn affinity to the induced Cd-binding compounds. Cadmium-binding peaks were never detected in control cultures. Results obtained for *U. confervicolum* on the SW column (Fig. 4) again confirmed the induction of only one Cd-binding compound. Its

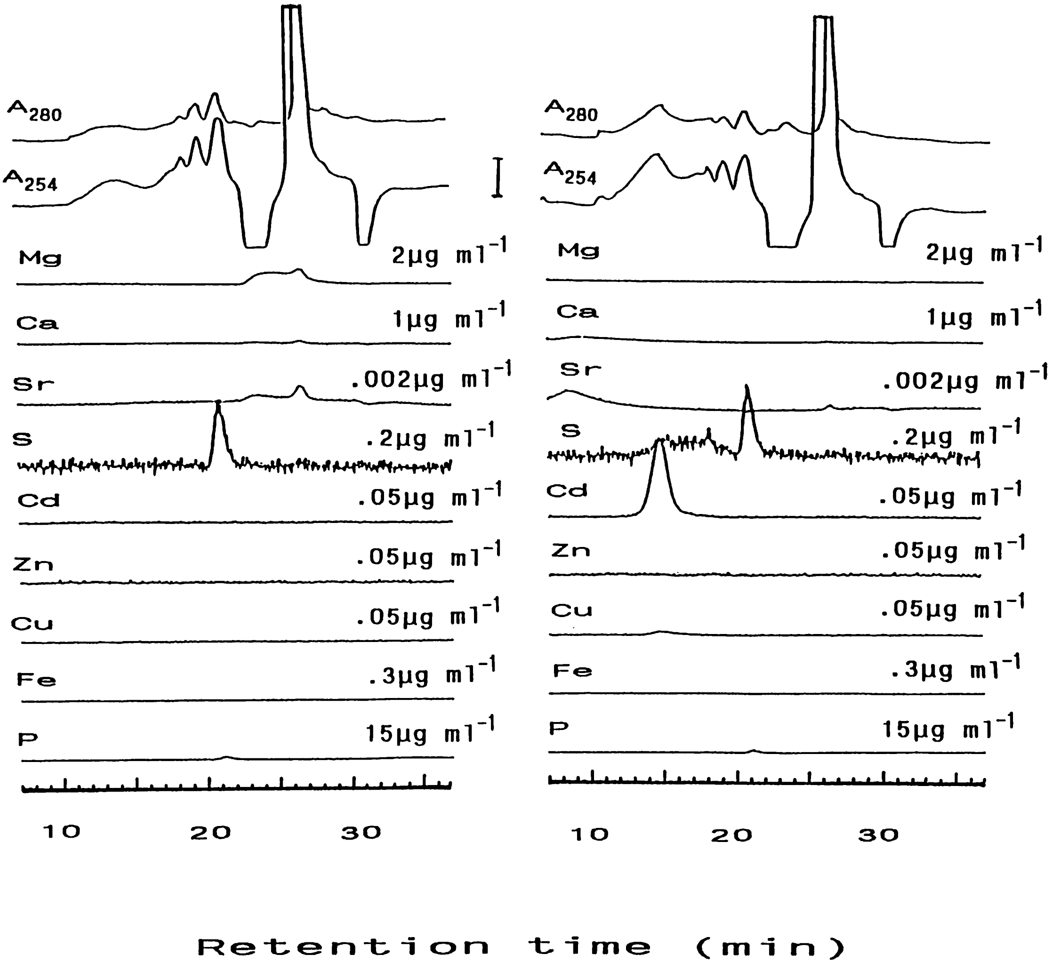

Fig. 4. HPLC-ICP profiles on a G3000SW column for the supernatants of *U. confervicolum*. Control cells (left) and cells exposed to 20 μM of Cd (right) for 3 weeks. Detector levels as in Fig. 3.

retention time (14.8 min) was shorter than that of the three Cd-binding fractions found in *C. vulgaris*.

Steffens (1990), analyzing the data on the occurrence of phytochelatins, concluded that the ability to synthesize phytochelatin in response to heavy metals is conserved from Orchidales, the most advanced group of higher plants, to the red, green, and brown algae. No other thiol-rich, heavy metal-binding compounds were detectable in the assayed plants, and phytochelatins synthesis was suggested as a generalized plant response to stress caused by heavy metals. Based on such assumption, we should not exclude that both strains examined in the present experiment formed phytochelatins with different numbers of γ-glutamyl-cysteine subunits, although the isolated Cd-binding compounds from *C. vulgaris* and *U. confervicolum* could have different characteristics. Wikfors *et al.* (1992) have reported recently that among five different Cd-tolerant algal species tested for Cd-binding polypeptides induction, only two of them, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* produced such compounds. Cd-tolerant strains of *Isochrysis galbana, Pavlova lutheri*, and *Tetraselmis maculata* did not produce detectable amounts of (γ-Glu-Cys)$_n$-Gly, what implies that other adaptive mechanisms may occur in some algae to ameliorate Cd stress. When influence of Cd on *Phaeodactylum tricornutum* was earlier analyzed by Kawaguchi and Maita (1990), two different Cd-binding peptides composed of glutamic acid, cysteine, and glycine were isolated. The chemical structure of these compounds was identical with phytochelatins induced in other algae and higher plants (Gekeler *et al.* 1988, Grill *et al.* 1987). The fact that *U. confervicolum* synthesized only one Cd-binding peak, while three Cd-peaks were found in *C. vulgaris*, suggests that the induction can be species-specific. The likelihood that metal stress in different algal species induces different specific adaptive mechanisms was earlier considered by Robinson (1989).

Shorter retention times of the Cd-binding compounds found in the Cd-treated algae compared to rat liver metallothionein-I and -II (Suzuki *et al.* 1987) obviously reflected their different chemical structure and composition. Metal-binding complexes isolated from plants are aggregates of heterogenous polypeptides and often behave like entities of 10–13.8 kDa in gel filtration media (Rauser 1990). Based on the determined structure and amino acid composition of phytochelatin isolated from *Rauvolfia serpentina*, Grill *et al.* (1987) concluded that the molecular weight of the native metal-containing phytochelatin complex was 2–4 kDa, rather than the 10 kDa often observed at low ionic strength.

As amino acid composition was not measured in the present study, the answer whether the isolated Cd-binding complexes should be classified as class II metallothioneins or phytochelatins remains too ambiguous, although some data support the latter possibility. Plants, opposite to animal species, always synthesize phytochelatins in response to heavy metals. However, Mehra *et al.* (1988) found that yeast *Torulopsis glabrata* exposed to Cu and Cd, formed both, metallothioneins and γ-glutamyl peptides for metal detoxification, and each system was regulated in metal-specific manner. Upon exposure to Cd, the cells synthesized only γ-glutamyl peptides. The coincidental synthesis of both above mentioned classes of compounds was never reported in algae or higher plants, but neither the technique applied in the present study nor methods recommended by Rauser (1991) or Grill *et al.* (1991) can resolve Cd-induced γ-glutamyl peptides and metallothioneins of the type found in *Torulopsis* (Mehra *et al.* 1988), *Saccharomyces cerevisiae* (Inouhe *et al.* 1991) or *Synechococcus* (Olafson *et al.* 1980).

A class II metallothionein isolated from metal tolerant aquatic insect, *Baetis thermicus* larvae was the heat-stable protein and most of other proteins in the supernatant were removable by heat-treatment without spoiling the metal binding capacity of metallothionein (Suzuki *et al.* 1988). Also the pea root (*Pisum sativum*) metallothionein produced in *E. coli* (Kille *et al.* 1991) seems to be a heat-stable protein. Heat-treatment to remove other "contaminating" proteins is commonly used in metallothionein purification procedures not only from animals but also from plant tissues (Rauser 1984, Rauser and Glover 1984). However, in the literature survey, we could not find any data on heat-stability of isolated metallothionein-like metal-binding complexes induced either in algae or in higher plants. In the present experiment, supernatants of Cd-exposed algae were heat-treated and the stability of metal-binding components was examined. Figure 5 illustrates HPLC-ICP profiles for heat-treated Cd-exposed algae obtained on the SW column. The isolated Cd-binding fractions were heat-stable components. The Cd, sulfur, and absorbance profiles did not change significantly after heat treatment (cf. Figs. 3–5). Minor changes in UV-profiles of Cd-exposed heat-treated supernatants were more likely observed in *C. vulgaris*. These results suggest a higher resistance to denaturation of the single Cd-binding component isolated from *U. confervicolum* compared with Cd-binding complex inducible in *C. vulgaris* and once more indicate different properties of metal-binding compounds induced in both algae observed.

Determination of amino acid composition of isolated Cd-binding compounds after their subsequent purification by reverse-phase

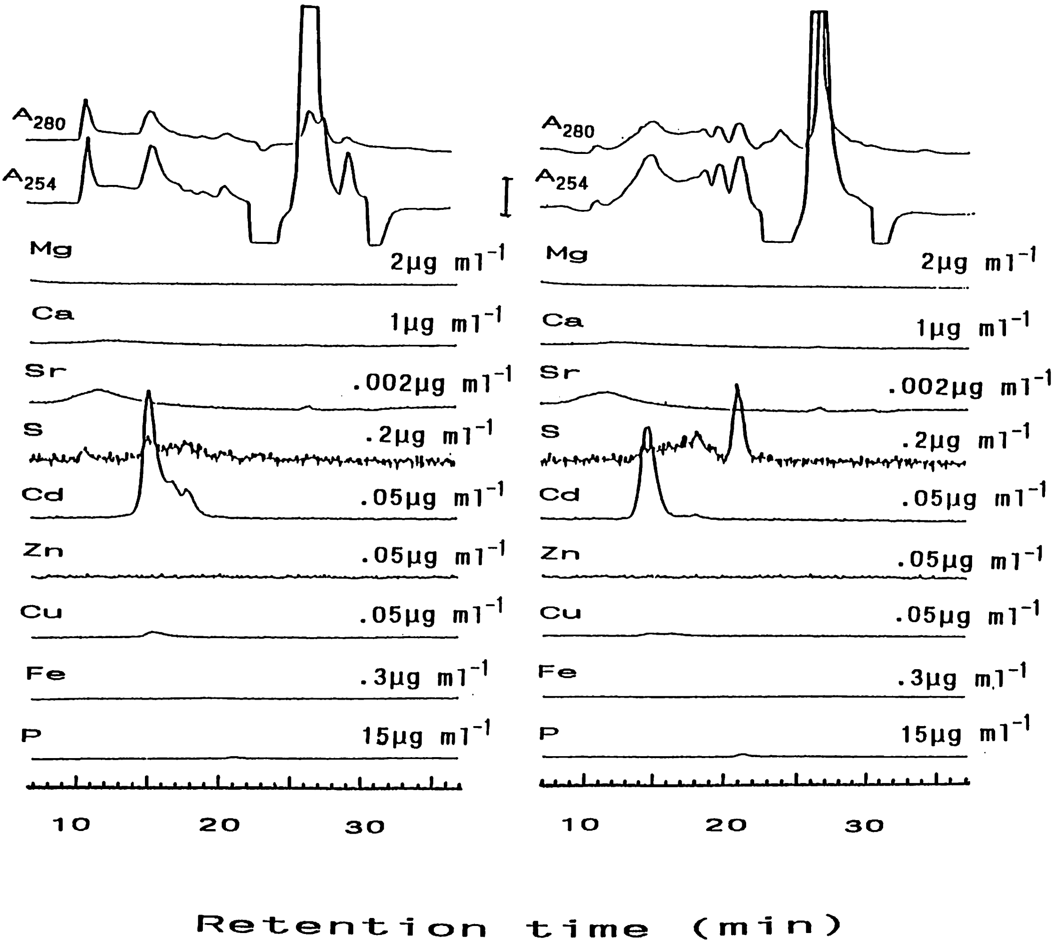

Fig. 5. Effect of heat-treatment on the distributions of elements in the supernatants of Cd-exposed algae. *C. vulgaris* (left) and *U. confervicolum* (right). Distribution profiles determined as in Fig. 3.

HPLC combined with thiol-rich compounds detection by Ellman's reagent will be a subject of our further experiments.

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Adam T. Wilczok・渡辺　信・川原早苗・鈴木和夫・菅原　淳：重金属耐性緑藻 *Chlorella vulgaris* と *Uronema confervicolum* による細胞内カドミウムの不活性化

重金属汚染河川から分離培養された緑藻*Chlorella vulgaris*と*Uronema confervicolum*がカドミウムの存在下で誘導する重金属結合ペプチドの分析を，高速液体クロマトグラフィー(HPLC)，原子吸光装置(AAS)及び誘導結合プラズマ発光分析計(ICP)を使って行った。*C. vulgaris*と*U. confervicolum*を20 μMの塩化カドミウムが添加された培地で3週間培養し，細胞内のカドミウムをAASで分析した結果，各々の細胞内には608 mg kg^{-1}及び597 mg kg^{-1}のカドミウムが蓄積されていた。双方とも蓄積されたカドミウムの約50%は，170000 gの遠心で上清の画分に存在していた。この画分について，HPLC-ICPのシステムで分析した結果，*C. vulgaris*には3種類のカドミウム結合ペプチドが，*U. confervicolum*には1種類のカドミウム結合ペプチドが確認された。これらの誘導されたペプチドは，いずれもイオウを多く含有していること，熱安定性であること，254 nmと280 nmでの吸収率比が高いことから，藻類や高等植物で誘導されているメタロチオネインと類似のペプチドであると思われる。

(305 茨城県つくば市小野川16–2　国立環境研究所)