Bull. Environ. Contam. Toxicol. (1989) 43:394–401

Amino Acid Composition of Cadmium–Binding Protein Induced in a Marine Diatom, *Phaeodactylum tricornutum**

Y. Maita and S. Kawaguchi

Division of Marine Biochemical Science, The Research Institute of North Pacific Fisheries, Faculty of Fisheries, Hokkaido University, Minato-cho 3-1-1, Hakodate, Hokkaido, 041, Japan

Organisms living in environments polluted with heavy metals develop tolerance against these contaminants. The tolerance has been attributed to the ability to synthesize metal binding substances; in animals these are called metallothioneins with molecular weights ranging from 6,000 to 7,000 (Kägi et al. 1984; Kägi and Kojima 1987), whereas in plants, a new class of metal binding proteins with molecular weights ranging from 1,800 to 4,000 called cadystin by Murasugi et al.(1981) or phytochelatin by Grill et al. (1985) has been isolated and characterized. These recent findings imply metal binding complexes from animals and plants, although having very similar functional properties, may have entirely different amino acid compositions.

Murasugi et al. (1981; 1984) reported that cadystin from fission yeast, Schizosaccharomyces pombe was composed of only glutamic acid, cysteine, and glycine. A year later, Grill et al. isolated a heavy metal binding substance from Rauwolfia serpetina which they called phytochelatin. This also contains only Glu, Cys, and Gly. Heavy metal binding complexes isolated from the water hyacinth (Fujita and Kawanishi 1987) and morning glory Datura innoxia (Jackson et al. 1984) also showed an amino acid composition similar to cadystin or phytochelatin.

In this study, the cadmium binding protein induced in the marine diatom, Phaeodactylum tricornutum, was isolated and purified and its amino acid composition determined.

Send reprint requests to Y. Maita at the above address.

*Contribution No. 207 from the Research Institute of North Pacific Fisheries, Faculty of Fisheries, Hokkaido University.

MATERIALS AND METHODS

Phaeodactylum tricornutum was batch-cultured in 5 L of Mutsu medium (Sato et al. 1973) at 20°C, with a daily photoperiod of 16 h light (3,000 lux) and 8 h dark. Cadmium as $CdCl_2$ was added to the experimental cultures, resulting in a final concentration of 1 mg/L. After 14 days, the cells were collected by centrifugation (1,000xg, 15 min), washed with filtered Cd-free sea water, and then resuspended in 20 mM phosphate buffer, pH 8.0 containing 0.25 M glucose and 0.1 M NaCl. Cell suspensions were homogenized three times, each at 2-min exposures using an Ultra Turrax Homogenizer (FRG). Homogenates were centrifuged at 12,000 x g for 30 min followed by 105,000 g for 60 min.

After centrifugation, the supernatant was chromatographed on a Sephadex G-75 (Pharmacia, Sweden) column (2.6 X 60 cm) which had been equilibrated with phosphate buffer. The same buffer was used for elution at a flow rate of 25 $mL \cdot h^{-1}$ and 5 mL fractions were collected. The fractions denoted by the bar (Fig. 1A) were pooled and applied to a DEAE Sephadex A-25 (Pharmacia) column (1 X 4 cm), equilibrated and washed with the buffer. Elution was done by step-wise method with six concentrations of NaCl in phosphate buffer solutions as shown in Fig. 2. Cadmium-rich fractions (Fig. 2) were then pooled and desalted on a Sephadex G-25 (Pharmacia) column (2.5X25cm), equilibrated with 0.2 M ammonium formate buffer, pH 8.0. Purified fractions were collected and freeze-dried to remove the ammonium formate. Ultraviolet absorbance of the fractions from both Sephadex G-75 and DEAE Sephadex A-25 columns were monitored at 254 and 280 nm with a spectrophotometer(Hitachi Seisakusho Co. Model 200-20). Cadmium concentration of the fractions was determined by using an atomic absorption spectrophotometer (Nippon Jarrel Ash Co. Model AA-782) with background absorbance correction of deuterium lamp. Standards for cadmium determinations were prepared from reagent grade $CdCl_2 \cdot 1/2H_2O$ (Wako Pure Chemical Industries, Japan).

Freeze-dried samples were oxidized with performic acid and then hydrolyzed in 6N HCl at 110°C for 18 h. Amino acid composition was analysed using an amino acid analyzer (Hitachi Seisakusho Co. Model 835). Amino acid standard solution, type H (Wako Pure Chemical Industries) was injected several times giving a coefficient of variation of ± 5%.

RESULTS AND DISCUSSION

The elution profile on Sephadex G-75 of the extract from cells cultured in cadmium-free medium is shown in

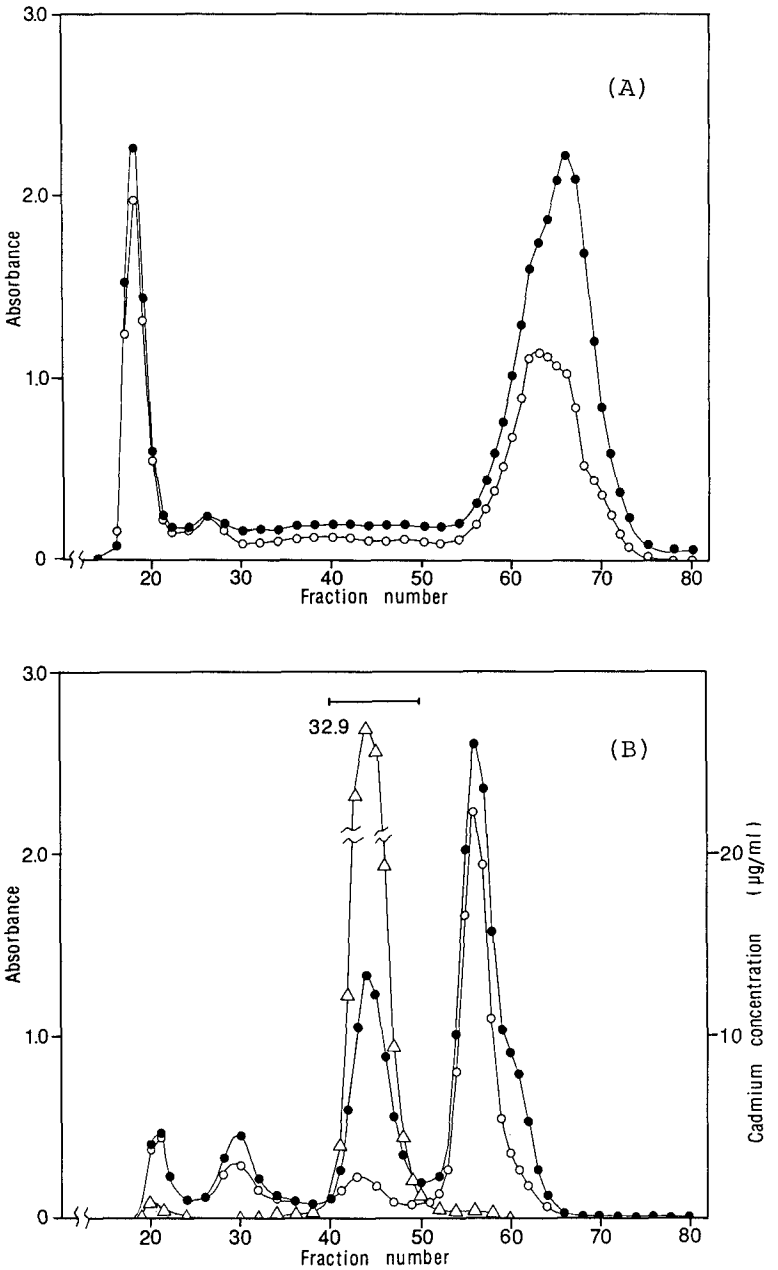

Figure 1. Sephadex G-75 gel-filtration profile of the cytosol fractions from the non-treated (A) and cadmium treated (B) cultures of Phaeodactylum tricornutum. The absorbance at 254 nm (—●—) and 280 nm (—○—), and the cadmium concentration (—△—) of each fraction (5mL) are indicated. The fractions denoted by the bar were pooled and purified further.

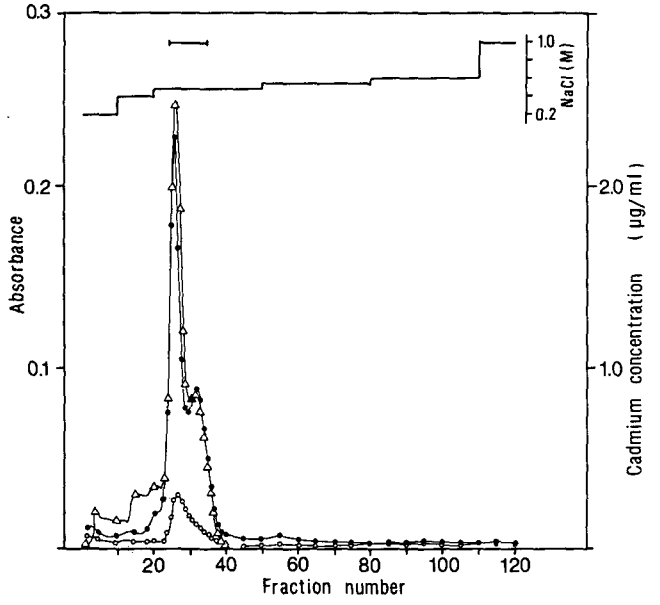

Figure 2. Elution profile of the cadmium-rich fractions from Sephadex G-75 gel filtration (see Fig. 1) on DEAE Sephadex A-25 column. The column was washed with 0.02M glucose before the stepwise elution with 0.4, 0.5, 0.55, 0.6 and 1.0 NaCl. The absorbance at 254 nm (—●—), 280 nm (—○—), and the cadmium concentration (—△—) of each fraction (5mL) are indicated. The fractions denoted by the bar were pooled, desalted, and analysed for amino acid content.

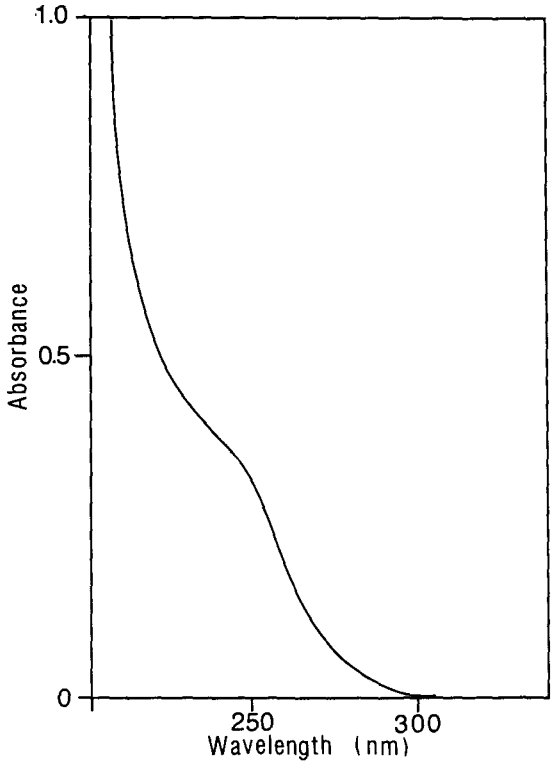

Figure 3. Ultraviolet absorption spectrum of fraction 26 from DEAE Sephadex ion exchange chromatography.

Fig. 1A. Two peaks corresponding to high and low molecular weight groups were observed. On exposure to 1 mg/L Cd for 14 days, four peaks resulted (Fig. 1B). Determination of cadmium in the fractions showed that the maximum concentration coincided with the third peak. The cadmium concentration associated with Peak III represented 95 % of the total cadmium content of the supernatant applied to the column. Peak III showed the highest 254 nm/280 nm absorbance ratio, implying a negligible amount of aromatic amino acids. These results showed that a heavy metal binding protein can be induced by cadmium in the marine diatom, Phaeodactylum tricornutum.

Elution profile after further purification by DEAE Sephadex A-25 is presented in Fig.2. Elution with different concentrations showed that most of the compounds were eluted with 0.5 M NaCl. Both the profiles of the absorbance at 254 nm and the cadmium concentration showed a peak with a shoulder. Amino acid analysis of the compound revealed that it was mainly composed of glutamic acid, cysteine, and glycine in a molar ratio of 4:3:1, with negligible amounts of other amino acids (Table 1). Since the recovery rate of Cys in the samples were variable, the amount of Cys might have been underestimated. Moreover, attempts were made to determine the amino acid sequence by Edman degradation using protein sequencer (Applied Biosystems, Model 477A), but it was not successful.

Table 1. Amino acid composition of cadmium-binding protein purified from cadmium rich fraction of Phaeodactylum tricornutum.

Amino acid	nmol/sample	mol %
Asp	0.91	3.5
Thr	0.38	1.5
Ser	0.66	2.6
Glu	10.38	40.3
Gly	2.45	9.6
Ala	1.01	3.9
Cys	7.59	29.5
Val	0.48	1.9
Ile	0.25	1.0
Leu	0.47	1.8
Phe	0.23	0.9
Lys	0.49	1.9
His	0.11	0.4
Arg	0.30	1.2
Pro	nd*	-
TOTAL	25.71	100.0

* Not detected

Ultraviolet absorbance spectrum of Fraction 26 showed a distinct shoulder at 245 nm (Fig. 3). This is identical to the spectrum obtained for Cd-BP2 but not for Cd-BP1 of fission yeast (Murasugi et al. 1983).

Phytochelatin purified from Rauwolfia serpentina has an amino acid sequence of $(\gamma\text{-Glu-Cys})_n$ Gly (n=3-7) (Grill et al. 1985) which was also similar to cadystin of fission yeast (Murasugi et al. 1984). Amino acid analysis of the cadmium-binding peptide isolated from several higher plants such as maize (Rauser and Glover 1984), water hyacinth (Fujita and Kawanishi 1986), stone parsley, soybean, sunflower, sweet potato, and adlay (Fujita and Kawanishi 1987) showed the presence of glutamic acid, cysteine, and glycine with negligible amounts of other amino acids. Hence, Fujita and Kawanishi (1987) suggested that the metallothionein-like cadmium binding compounds described previously for higher plants should be reexamined.

Nagano et al. (1982 a and b) reported a cadmium-binding complex in the freshwater unicellular algae, Chlorella ellipsoidea. Although the amino acid composition was not determined, its highly anionic behavior on the ion exchange column suggests a high content of dicarboxylic amino acids, probably glutamic acid. Hence, it might be a complex similar to the cadmium-binding proteins present in fission yeast. In another freshwater unicellular algae, Euglena gracilis, the cadmium-binding peptides showed similar characteristics to Cd-BP1 of fission yeast (Weber et al. 1987).

The isolation of cadmium-binding peptide similar to that present in fission yeast suggests that the synthesis of the peptide can be induced not only in terrestrial higher plants and freshwater unicellular algae but also in the diatom, P. tricornutum.

Since the amount of cyteine in the samples is usually underestimated as mentioned above, the ratio of Glu:Cys:Gly in P. tricornutum might be 4:4:1 instead of 4:3:1. The amino acid composition would suggest that the cadmium-binding peptide induced in the marine diatom is similar to that of higher plants and fission yeast.

Results of the preliminary study (Maita et al. 1988) using Sephadex G-75 to isolate the cadmium-binding complex induced in P. tricornutum suggested that the compound was similar to metallothionein. They described the substance metallothionein-like protein. However, in the present study, the UV absorption spectrum (Fig. 3) and amino acid analysis (Table 1) of the samples purified by DEAE Sephadex A-25

suggested a similarity to Cd-BP2 of fission yeast. As far as the authors know, this is the first report on the characterization of a cadmium-binding protein induced in a marine diatom. Further investigations are being conducted to characterize the cadmium-binding peptide and the results will be published later.

Acknowledgments. We thank Dr. M. Fujita, Faculty of Agriculture, Kagawa University for his advise and discussion; Director H. Sawada and staffs of Hakodate City Institute of Public Health for their technical support; Ms. L. V. Castillo for reading the manuscript. This work was supported by the Steel Industry Foundation for the Advancement of Environmental Protection Technology, No. 59-2-22.

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Received September 12, 1988; accepted February 10, 1989.