Bull. Environ. Contam. Toxicol. (1990) 45:893–899

Bulletin of Environmental Contamination and Toxicology

Amino Acid Sequence of Cadmium-Binding Peptide Induced in a Marine Diatom, *Phaeodactylum tricornutum*

S. Kawaguchi and Y. Maita

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

Many studies conducted to date have shown that heavy metals have detrimental effects on organisms. However, many of them are resistant to certain amounts of those metals. In animals, a cystein rich unique protein, metallothionein, is known for its ability to detoxify heavy metals (Kägi 1974), whereas in higher plants, a new class of metal binding peptide, phytochelatin (Grill et al. 1985) has been characterized. In general, the effects of heavy metals on terrestrial organisms are better known than the effects on aquatic organisms. Although in the case of freshwater algae, several studies have shown the processes of metal detoxification (e.g. Weber et al. 1987; Watanabe et al. 1988), only a few studies have shown the processes of metal detoxification in marine phytoplankton. However in our previous studies, cadmium binding peptide which was similar to phytochelatin, was purified from marine diatom cultured under cadmium rich condition (Maita et al. 1988; Maita and Kawaguchi 1989). In this paper, the amino acid sequence of Cd binding peptide was predicted.

MATERIALS AND METHODS

Phaeodactylum tricornutum was batch-cultured in 0.5-5 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, copper, and zinc were added to the experimental cultures, resulting in a final concentration shown in the figures. After 14 days, the cells were collected by centrifugation (1,000 x g, 15 min), washed with filtered metal-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 x g for 60 min.

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

After centrifugation, the supernatant was chromatographed on a Sephadex G-75 (Pharmmacia, 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. 1) 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 0.1-1.0 M NaCl in phosphate buffer solutions. Cadmium-rich fractions (Fig. 2) were then pooled and desalted on a Sephadex G-25 (Pharmacia) column (2.5 X 25cm), equilibrated with 0.2 M ammonium formate buffer, pH 8.0. In the case of a shoulder peak, desalting was performed after rechromatography on the DEAE Sephadex A-25 under the same conditions. Ultraviolet absorbance of the fractions from both Sephadex G-75 and DEAE Sephadex A-25 columns was 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 metal determinations were prepared from reagent grade $CdCl_2 \cdot 5/2H_2O$, $CuSO_4 \cdot 5H_2O$, and $ZnCl_2$ (Wako Pure Chemical Industries, Japan) in 0.1 N HNO_3.

Purified fractions were collected, freeze-dried, and oxidized with performic acid according to the method of Moore (1963) for the sequence analysis. Amino acid located in the N-terminal of the peptide was dansylated (Hartley and Massey 1956). DNS-amino acid was determined by comparing amino acid composition before and after dansylation. The amino acid which apparently decreased on amino acid analysis was defined as the DNS-amino acid. In order to determine the C-terminal amino acid, hydrazinolysis (Niu and Fraenkel-Conrat 1955) was performed.

Amino acid composition was analysed using an amino acid analyzer (Shimadzu Seisakusho Co. Model LC-5A). Amino acid standard solution, type H (Wako Pure Chemical Industries) was injected several times giving a coefficient of variation of $\pm$ 5%.

RESULTS AND DISCUSSION

The elution profile on Sephadex G-75 of the extract from cells cultured under the condition of 1 mg/L Cd is shown in Fig. 1. Cd was eluted as a single peak. On the other hand, induction of binding peptides by individual additions of sublethal concentrations of Cu or Zn was not observed. However, addition of Cu and Zn to plankton growing in the presence of Cd caused the

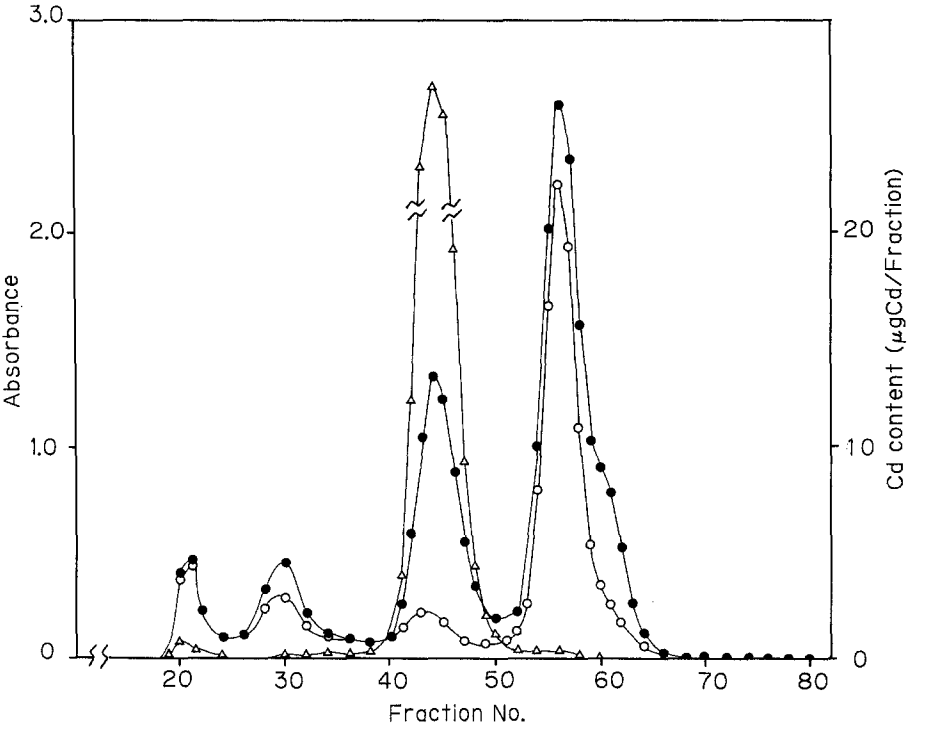

Figure 1. Sephadex G-75 gel filtration profile of the cytosol fractions from the culture of P. tricornutum treated in the medium containing 1 mg/L Cd. Absorbance at 254 nm (—●—), 280 nm (—○—), and Cd concentration (—△—). Fractions denoted by the bar were pooled and purified further.

displacement of Cd in the binding peptide fraction with Cu (Fig. 2). This suggests that the peptide has a higher affinity for Cu than Cd and Zn. Similar results are also shown by Jackson et al. (1987) in higher plants.

Results of the further purification on DEAE Sephadex A-25 is shown in Fig. 3. Elution with different concentrations of NaCl showed that most of the compounds were eluted with 0.5 M NaCl. Both the profiles of the absorbance at 254 nm and the Cd concentration showed a peak (CdBP-1) with a shoulder (CdBP-2).

CdBP-1 and CdBP-2 consisted of only three species of amino acid, Glu, Cys, and Gly. The Glu/Cys/Gly ratio for CdBP-1 was 4/3.4/1. The HPLC chromatogram of CdBP-1 after hydrazinolysis (Fig. 4) clearly shows that the amino acid located in the C-terminal is Gly.

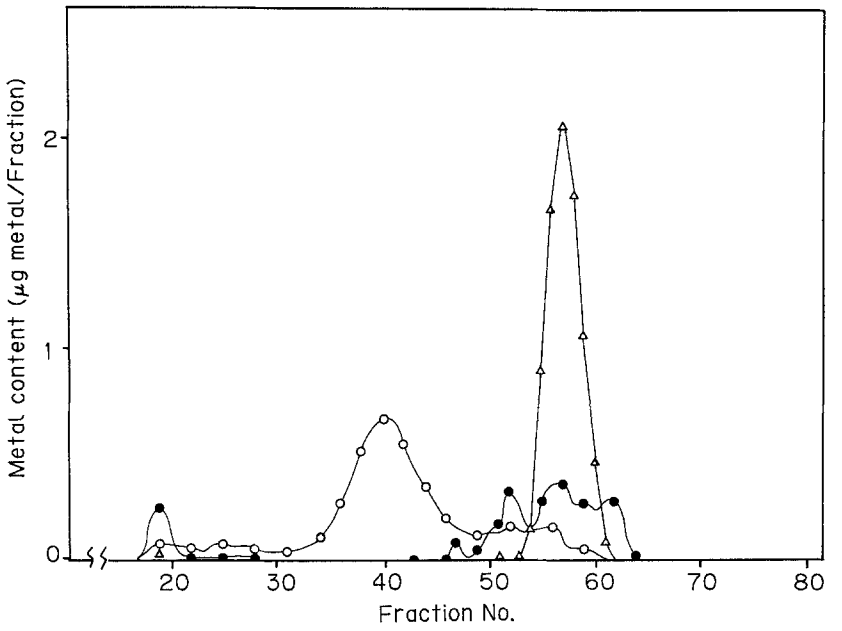

Figure 2. Sephadex G-75 elution profile of the supernatant fraction prepared by ultracentrifugation (105,000 g) from homogenized P. tricornutum cultured for 10 days under coexistence of 0.1 ppm Cd (—△—), Cu (—○—), and Zn(—●—).

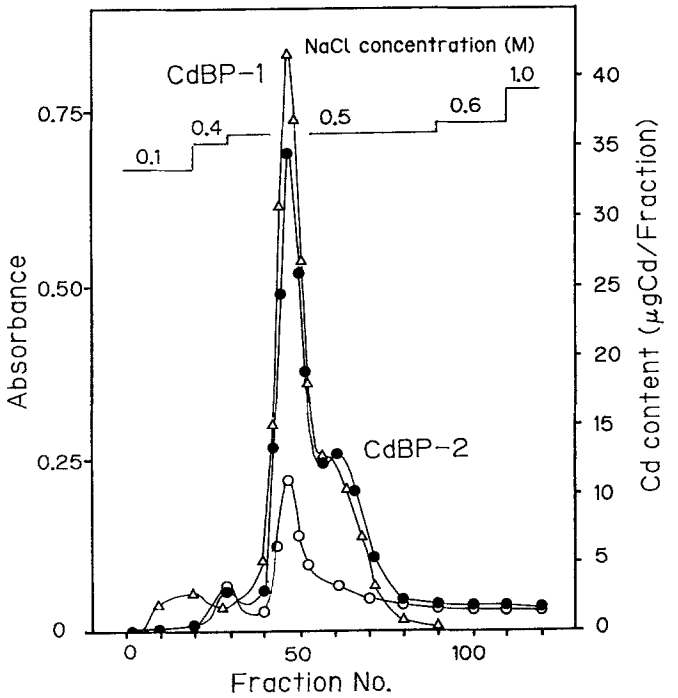

Figure 3. Ion exchange chromatogram (DEAE Sephadex A-25) of Cd rich fraction obtained by gel filtration. The symbols are same as Fig. 1.

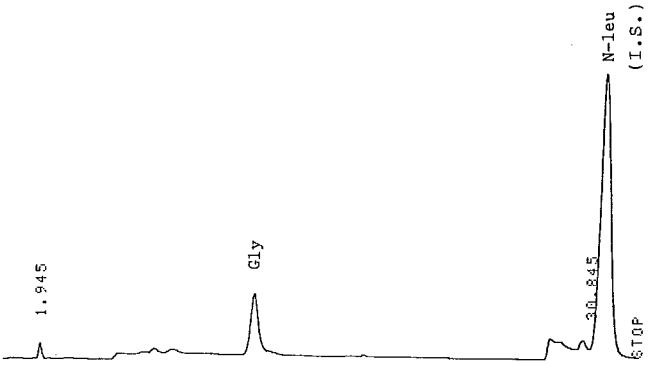

Figure 4. HPLC chromatogram of CdBP-1 after hydrazinolysis. N-leu was added for the internal standard.

Table 1 shows the amino acid values obtained from the injection of 5.6 nmol peptide. This result shows that Gly/peptide ratio is 1. Therefore, the peptide contains only one gly, which is located in the C-terminal position. The Result of dansylation of CdBP-1 is summarized in Table 2. Amino acid composition of CdBP-1 before and after dansylation of N-terminal amino acid resulted in the decrease of Glu at the rate of 0.7 mol/mol peptide. In the case of glutathione (a peptide which has Glu in the N-terminal), there was a decrease of Glu at the rate of 0.7 mol/mol peptide after the dansylation, as shown in Table 3 . These results clearly indicate that Glu exists in the N-terminal of CdBP-1. From the previous study according to the Cd binding peptide in P. tricornutum, Edman degradation was not successful (Maita and Kawaguchi 1989). This phenomenon strongly suggests the possibility of γ-Glu residue (Grill et al. 1985). Based on all the characteristics of the purified CdBP-1 in this study, we concluded that CdBP-1 is identical with phytochelatin, $(\gamma\text{-Glu-Cys})_4\text{-Gly}$ (Grill et al. 1985).

Table 1. Molar quantities (nmol/sample) of the peptide (CdBP-1) and amino acids of hydrolyzed peptide. The values were determined by using HPLC, OPA method.

Sample	Peptide or amino acid, nmol/sample			
	BP-1	Glu	Cys	Gly
CdBP-1 direct	5.6	-	-	-
CdBP-1 hydrolyzed	-	23.2	18.0	5.5

Table 2. Amino acid composition (mol/peptide) of CdBP-1 and dansylated CdBP-1 (DNS CdBP-1).

Sample	Amino acid, nmol/peptide		
	Glu	Cys	Gly
a) CdBP-1	4.0	2.9	1.0
b) DNS CdBP-1	3.3	2.8	1.0
a)-b)	0.7	0.1	-

Table 3. Amino acid composition (mol/peptide) of glutathione(GSH) and dansylated glutathione.

Sample	Amino acid, mol/peptide		
	Glu	Cys	Gly
a) GSH	0.86	0.74	1.0
b) DNS GSH	0.17	0.75	1.0
a)-b)	0.69	-	-

Table 4. Glu/Gly ratios of Cd binding peptides in P. tricornutum.

Peptide	Glu/Gly ratio
Fr-2*	2.0
Fr-3*	3.3
CdBP-1	4.0
CdBP-2	5.2

*: Cadmium peptide fractions obtained in our previous study (Maita et al. 1988).

Table 4 shows the Glu/Gly ratios of Cd-binding peptide purified from P. tricornutum. The values of Fr-2 and Fr-3 were calculated from our previous study on the same plankton (Maita et al. 1988). Ratios of 2, 3, 4, and 5 were obtained. These values indicate the existence of the peptides which have the sequence of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n=2 to 5). In higher plants (Grill et al. 1987), peptides which have the n value from 2 to 11 were observed, and suggested that the peptides are synthesized by sequental addition of γ-glutamyl cystein residues to glutathione. The presence of cadmium

binding peptides which have the chain length of n=2 to 5 in the marine diatom may also suggest that the peptides are produced through the same process.

Acknowledgments. We thank Dr. M. Fujita, Faculty of Agriculture, Kagawa University for his advise and discussion. A part of this work was presented in PACIFICHEM '89 in Hawaii. This is also contribution No. 226 from the Research Institute of North Pacific Fisheries, Hokkaido University.

REFERENCES

Grill E, Winnacker EL, Zenk MH (1985) Phytochelatins: The principal heavy-metal complexing peptides of higher plants. Science 230:674-676

Grill E, Winnacker EL, Zenk MH (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc Natl Acad Sci USA 84:439-443

Hartley BS, Massey V (1956) The active centre of chymotrypsin I. Labelling with a fluorescent dye. Biochim Biophys Acta 21:58-70

Jackson PJ, Unkefer CJ, Doolen JA, Watt K, Robinson NJ (1987) Poly(γ-glutamylcysteinyl)glycine: Its role in cadmium resistance in plant cells. Proc Natl Acad Sci USA 84:6619-6623

Kägi JHR, Hinmelhoch SR, Whanger PD, Bethune JL, Vallee BL (1974) Equine hepatic and renal metallothioneins. J Biol Chem 249:3537-3542

Maita Y, Kawaguchi S, Tada K (1988) A metallothionein-like protein induced in a diatom, Phaeodactylum tricornutum. In: Okaichi T, Anderson DM, Nemoto T (eds) Red tide: Biology, environmental science and toxicology, Elsevier, New York, pp 345-348

Maita Y, Kawaguchi S (1989) Amino acid composition of cadmium binding protein induced in a marine diatom Phaeodactylum tricornutum. Bull Environ Contam Toxicol 43:394-401

Moore S (1963) On the determination of cysteine as cysteic acid. J Biol Chem 238:235-237

Niu CI, Fraenkel-Conrat H (1955) Determination of C-terminal amino acids and peptides by hydrazinolysis. J Amer Chem Soc 77:5882-5885

Sato A, Yokoyama K, Naoe H (1973) Akagai no shubyou seisan (in Japanese). Aomori Prefecture Ann Rep 2: 79-90

Watanabe M, Takeuchi Y, Takamura N (1988) Copper tolerance of benthic diatom Achnanthes minutissima. Res Rep Natl Inst Environ Stud Jpn 114:233-232

Weber DN, Shaw CF, Petering DH (1987) Euglena gracilis cadmium-binding protein-II contains sulfide ion. J Biol Chem 262:6962-6964

Received December 18, 1989; accepted April 9, 1990.