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Cadmium-Binding Proteins in the Mussel, *Mytilus edulis*

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Inducible cadmium-binding proteins (Cd-BP) in the mussel, *Mytilus edulis*, were resolved into two molecular weight components, designated Cd-BP_{10} and Cd-BP_{20}, by gel-permeation chromatography on Sephadex G-75. Each of these two molecular weight components were further resolved into four subcomponents by DEAE–ion-exchange chromatography. All eight subcomponents bound cadmium and exhibited significant UV absorption at 254 nm and little absorption at 280 nm. Each subcomponent was purified and subjected to amino acid composition analysis. Two classes were identified, one having higher cysteine (23.9–26.6 mole-%) and lower glutamic acid contents compared to the other class (11.6–18.2 mole-% cysteine). All subcomponents have a relatively high glycine content (approximately 15 mole-%) relative to mammalian metallothioneins (approximately 8 mole-%). Although the Cd-BP_{20} have apparent molecular weights almost twice the Cd-BP_{10}, the exact molecular relationship between these binding proteins is not known.

Introduction

In recent years several investigators have reported the occurrence of metal binding proteins (BP) in the mussel *Mytilus edulis* as well as *Myutilus galloprovincialis*. Much of this research was stimulated by the original paper of Noel-Lambot which appeared in 1976 (*1*). This pioneering work on mussels clearly defined some of the unique properties of mussel BPs. First, when induced in the laboratory by exposure to cadmium in sea water, there are two BP components resolvable by gel-permeation chromatography on Sephadex G-75 columns. One component has an apparent molecular weight similar to that of mammalian metallothioneins (MT) while the second has a significantly larger apparent molecular weight. Secondly, the Cd-BP obtained had a high percentage of cysteine and an absorption spectrum typical of mammalian MT. Third, the proteins induced in the laboratory contained very low levels of zinc and copper. Soon after the Noel-Lambot report, Talbot and Magee (*2*) demonstrated that similar proteins were present in mussels exposed to contaminating metals in the natural environment. More recent studies have verified the presence of BPs in *Mytilus edulis* following exposure to cadmium (*3–5*) and mercury (*6*). In a related species, *Mytilus galloprovincialis*, BPs have also been demonstrated following exposure to both cadmium (*7*) and copper (*8–10*).

In spite of the interest in the mussel metal BPs, relatively little research has been directed toward a careful characterization of the proteins themselves. George et al. (*3*) isolated three subcomponents of the higher molecular weight BP (apparent MW = 25,000 ± 5000) on DEAE–cellulose anion-exchange chromatography. Amino acid composition for these proteins (Table 1) indicated a significantly lower value for the cysteine content than mammalian MT. The investigators suggest that the BPs of higher molecular weight may be dimers of the lower molecular weight component, although no characterization of the smaller BPs is given. Frankenne et al. (*11*) also attempted to characterize the mussel BPs. Unfortunately, they did not resolve the two molecular weight BP components on Ultrogel AcA 54, and thus the material used for amino acid composition studies was a mixture of various components as indicated by their PAGE results. In spite of this, they obtained a very high cysteine content (25.5 mole-%) (Table 1). The only other study characterizing mussel BPs is that of Roesijadi and Hall (*6*) following induction with mercury. These investigators were able to obtain a protein(s) which exhibited multiple bands on PAGE and had an amino acid composition with relatively low cysteine content (7.8%) and a large molecular weight (14,400).

The data reported here are the results of an attempt to further characterize the nature of the cadmium BPs in the mussel, *Mytilus edulis*, following induction by cadmium under laboratory conditions. In this study both molecular weight classes of induced Cd-BPs are explored.

Materials and Methods

Adult mussels, *Mytilus edulis*, were obtained from either the North Esk or Ythan estuaries (Scotland) and maintained in sea water aquaria at the Institute of Marine Biochemistry, Aberdeen, Scotland. Mussels were exposed to 100 μg Cd^{2+}/L at 10°C for 3 to 4 months. Water

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Table 1. Summary of reported amino acid compositions for mussel (*Mytilus edulis*) metal BPs.

	Amino acid composition, mole-%[a]					
	MT-1	MT-2	MT-2b	MT-3		
	(*3*)	(*3*)	(*3*)	(*3*)	(*11*)	(*6*)
Cys	11.2	17.6	20	6.3	25.5	7.8
Asx	10.8	9.5	9.1	12.3	8.1	12.8
Thr	8.0	9.5	6.3	8.1	6.4	7.0
Ser	7.6	7.5	6.5	8.2	7.9	7.3
Glx	8.9	7.6	5.8	7.8	4.5	12.2
Pro	4.9	4.4	3.8	5.2	6.8	5.6
Gly	11.7	12.1	18.6	10.8	15.8	9.4
Ala	5.9	5.9	4.7	5.0	5.2	5.8
Val	4.3	3.9	4.2	3.5	3.8	5.4
Met	1.4	tr	—	tr	<0.5	1.2
Ile	3.7	2.6	3.5	2.7	4.0	4.7
Leu	4.7	4.3	3.0	2.8	1.3	5.7
Tyr	1.7	1.2	—	0.6	<0.5	2.3
Phe	2.3	2.1	—	1.0	1.3	2.8
His	1.5	1.2	—	3.4	<0.5	0.8
Lys	9.5	10.1	11.5	9.8	7.7	6.5
Arg	2.4	2.3	1.2	1.6	1.8	2.5

[a] Compositions as reported in the literature (*3,6,11*).

was changed three times per week and cadmium dosing was repeated at each water change. Following exposure, mussels were shucked in batches of 30 to 50 organisms and the soft tissues were dissected to exclude adductor muscle and foot. Tissues (50–100 g) were homogenized in a volume of distilled water equal to the tissue weight. The homogenate was centrifuged at 79,000*g* for 120 min and the resulting supernatant filtered and collected for further processing.

Cadmium binding proteins were purified by the following procedure. Mussel cytosol was diluted 1:20 with distilled water and the pH adjusted to 8.5 with 0.1 N NaOH. DEAE–cellulose (Whatman DE52) was added (100 mL of swollen gel equilibrated with 0.02 M Tris, pH 8.5). The mixture was stirred at low speed for 2 hr with N_2 constantly bubbled through the solution. The DEAE–cellulose was recovered by filtration and washed with 0.02 M Tris, pH 8.5, on the filter, transferred to a beaker and extracted with 100 mL of 0.5 M Tris, pH 7.5, for 2 hr. The mixture was refiltered, the DEAE washed with an additional 50 mL of extraction buffer and the filtrate and wash were pooled for the next step. The pH was readjusted to 8.5 with NaOH.

The DEAE extract (approximately 130 mL) was applied to a Sephadex G-75 column (5 × 90 cm) and eluted with 2 L of 0.02 M Tris, pH 8.5, at a flow rate of 120 mL/hr. Fractions of 10 mL volume were collected and analyzed for cadmium by atomic absorption spectrophotometry without sample dilution. The major cadmium binding component was resolved into two overlapping peaks. The fractions constituting the leading portion of the higher molecular weight peak and the trailing portion of the lower molecular weight peak were each collected and designated Cd-BP_{10} and Cd-BP_{20}, respectively (the subscripts refer to crude estimates of molecular weights).

The lower molecular weight component, Cd-BP_{10}, was

Table 2. Amino acid composition of *Mytilus edulis* cadmium-binding proteins.

	Amino acid composition, residue-%							
	Cd-BP_{10}				Cd-BP_{20}			
Amino acid	10I	10II	10III	10IV	20I	20II	20III	20IV
Cys	23.9	15.9	26.4	11.6	26.1	17.0	18.2	26.2
Asx	7.7	7.5	8.4	9.8	7.9	8.5	13.3	9.0
Thr	5.9	5.4	7.1	7.4	7.5	7.1	9.2	7.1
Ser	7.9	14.6	8.0	8.4	7.9	10.7	7.2	7.9
Glx	5.7	10.3	4.6	10.5	4.1	8.8	8.0	5.6
Pro	5.6	5.1	4.4	5.9	4.8	5.1	3.4	4.8
Gly	15.1	17.5	17.2	14.3	18.2	16.6	14.8	14.8
Ala	6.2	7.7	5.2	7.9	3.8	5.9	10.3	5.4
Val	4.8	4.1	4.8	6.1	4.8	4.9	5.1	4.7
Ile	2.8	2.5	3.3	3.1	3.0	3.0	3.1	3.6
Leu	1.2	1.0	0.8	4.8	0.5	2.1	3.1	1.5
Phe	—	—	—	1.2	—	.07	—	trace
Ilis	—	1.6	—	—	trace	1.3	—	trace
Lys	9.7	6.4	7.9	5.9	10.0	6.7	4.6	7.2
Arg	2.6	0.7	1.6	3.0	1.5	1.5	n.d.	1.6

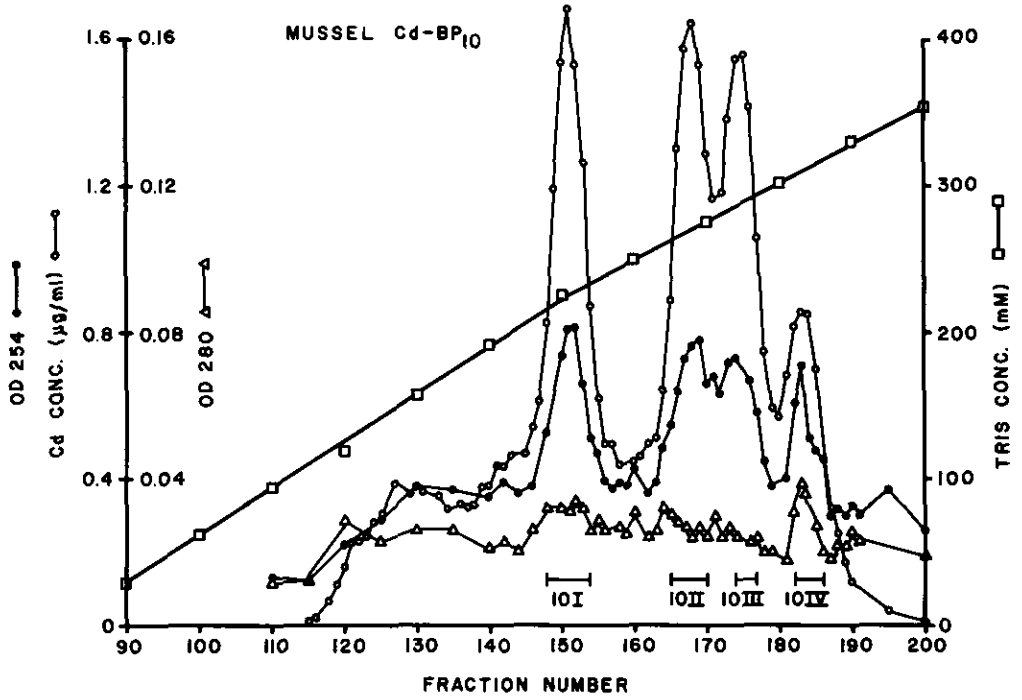

FIGURE 1. Anion exchange chromatography of Cd-BP_{10}. Following chromatography on Sephadex G-75, Cd-BP_{10} was applied to a DEAE–anion-exchange column (Whatman DE52, 2.5 × 12 cm) and eluted with a linear gradient generated by 250 mL each of starting buffer (0.02 M Tris, pH 8.5) and final buffer (0.4 M Tris, pH 8.5) at a flow rate of 36 mL/hr. Fractions (4 mL) were collected and analyzed for cadmium, by atomic absorption spectrophotometry, and UV absorbance at 254 and 280 nm.

pooled from several preparations and rechromatographed on Sephadex G-75 to remove any contaminating Cd-BP_{20}. Both cadmium-binding protein components, Cd-BP_{10} and Cd-BP_{20}, were chromatographed on DEAE–ion-exchange columns (60 mL bed volume, 2.5 × 12 cm). DEAE-cellulose (Whatman DE52) was equilibrated with 0.02 M Tris, pH 8.5, at 4°C prior to application of the sample. The column was loaded at 50 mL/hr and washed with 1 bed volume of starting buffer. A linear gradient was generated from 250 mL each of starting buffer and final buffer (0.4 M Tris, pH 8.5). The column was eluted at 36 mL/hr and 4 mL fractions collected. Fractions were

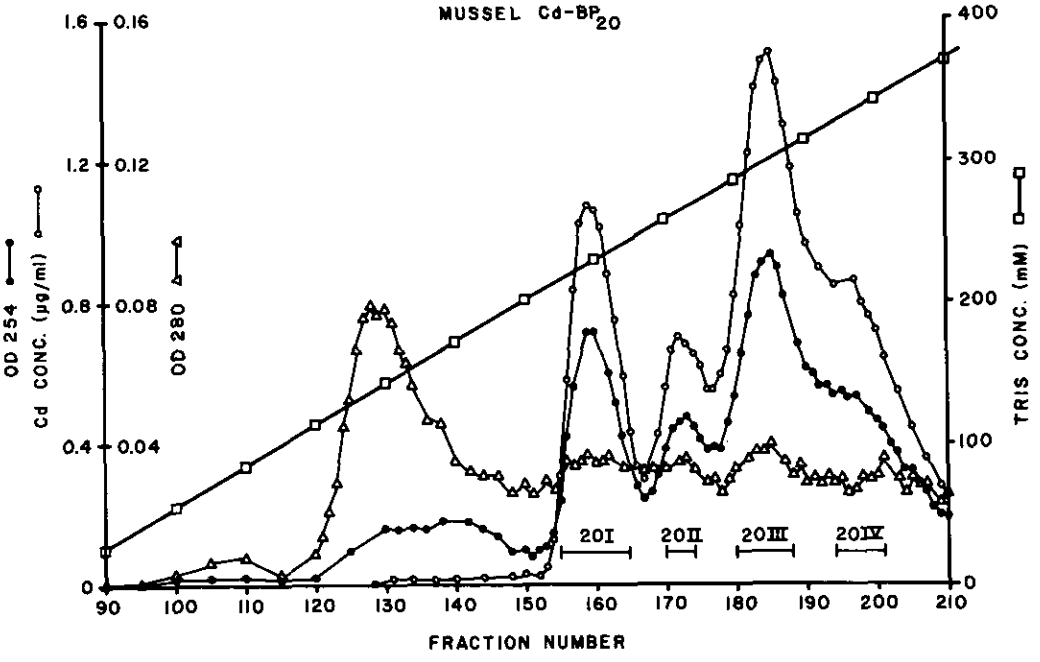

FIGURE 2. Anion exchange chromatography of Cd-BP_{20}. Experimental conditions are similar to Figure 1.

Table 3. Comparison of the amino acid compositions of *Mytilus edulis* Cd-BP_{10} (I) with binding proteins isolated from various organisms.[a]

	Amino acid compositions, mole-%			
	Mussel Cd-BP_{10}(I)	Oyster Cd-BP	Horse MT[b]	Human MT[c]
Cys	23.6	24.8	32.6	32.8
Asx	7.7	6.8	5.0	6.6
Thr	5.9	6.9	2.3	3.3
Ser	7.9	9.4	11.6	13.1
Glx	5.7	6.4	4.5	3.3
Pro	5.6	5.5	5.1	3.3
Gly	15.1	15.7	10.0	8.2
Ala	6.2	5.9	9.4	11.5
Val	4.8	2.3	2.5	1.6
Met	—	0.7	1.5	1.0
Ile	2.8	1.5	0.6	1.6
Leu	1.2	1.0	0.6	—
Try	—	0.6	—	—
Phe	—	0.5	—	—
His	—	—	—	—
Lys	9.7	11.3	10.4	13.1
Arg	2.6	0.6	2.2	3.3

[a] Data from Frazier and Brouwer (unreported).
[b] Data of Kägi et al. (*13*).
[c] Data of Kissling and Kägi (*14*).

analyzed for cadmium, OD_{254} and OD_{280}. The chromatograms of both Cd-BP components exhibited four cadmium peaks designated Cd-BP_{10}(I)–Cd-BP_{10}(IV) and Cd-BP_{20}(I)–Cd-BP_{20}(IV).

Each partially purified subcomponent was further purified. Only the central fractions of each peak were pooled and the pooled sample rechromatographed on DEAE under the conditions as described above. In all cases the rechromatography profiles now gave only one symmetrical peak with little evidence of contamination by other subcomponents. Fractions with cadmium concentration greater than 0.5 μg/mL were pooled. The individual subcomponents were desalted on a Sephadex G-50 column, frozen and lyophilized.

Amino acid composition of each subfraction was determined after performic acid oxidation on a Durrum D-500 Analyzer in Professor J. Kagi's laboratory (University of Zurich). Values for threonine and serine were corrected for 5 and 12.5% loss during hydrolysis, respectively.

Results

In a typical preparation the cadmium, zinc and copper concentrations in fresh mussel tissues used for the extraction of Cd-BPs were 31.3, 12.8, and 1.4 μg/g wet weight, respectively. Almost 60% of the cytosolic cadmium is associated with Cd-BP_{20}, the larger Cd-BP, and only 25% with Cd-BP_{10}. The remaining cytosolic cadmium was associated with high molecular weight macromolecules (MW> 50,000). Cadmium was the major metal associated with both BPs. Smaller amounts of zinc were bound to the BPs and only traces of copper were present.

Typical chromatographic profiles for Cd-BP_{10} and Cd-BP_{20} on DEAE are given in Figures 1 and 2, respectively. Four cadmium peaks are observed in each profile. Each peak exhibits significant UV absorption at 254 nm and relatively low absorption at 280 nm. The amino acid compositions of each of these subcomponents are given in Table 2. Subcomponents 10I and 10III have high cysteine and low glutamic acid residue contents compared to 10II and 10IV. All subcomponents have a relatively high glycine content (approximately 15 residue-%) compared to mammalian metallothioneins (8–10 residue-%). A similar pattern is observed for the higher molecular weight proteins (Cd-BP_{20}). Subcomponents 20I and 20IV have high cysteine and lower glutamic acid residue contents than 20II and 20III. All subcomponents have high glycine content. Subcomponents 10II and 20II both have higher serine content than other subcomponents and seem to be quite similar in composition.

An attempt to reduce Cd-BP_{20} (III) by the procedure described by Suzuki and Yamamura (*12*) to split metallothionein dimers gave inconclusive results. If reduction occurred, only a very small percentage of the higher molecular weight component was reduced to a smaller component.

Discussion

Careful anion exchange chromatography of mussel Cd-BPs reveal eight subcomponents, four Cd-BP_{10} and four Cd-BP_{20}. Subclasses of these proteins appear to exist based on cysteine content. The high cysteine content (23.9–26.6 residue -%) subcomponents (10I, 10III, 20I and 20IV) have generally similar amino acid compositions. The low cysteine content (11.6–18.2 residue -%) subcomponents are further subdivided by their serine contents into low cysteine–low serine subcomponents (10IV and 20III) and low cysteine–high serine subcomponents (10II and 20II). The functional role of the multiple molecular weight species and charge forms of the BP is not known.

The amino acid composition of one of the high cysteine subcomponents [Cd-BP_{10} (I)] is compared to several other cadmium/zinc binding proteins in Table 3. The oyster protein reported in this table was obtained in collaboration with Dr. Marius Brouwer (Duke University Marine Laboratory). The very close correspondence in amino acid composition is apparent and may indicate similar proteins in these two mollusk species. However, it should be noted that DEAE–anion-exchange chromatography indicated there was only one oyster Cd-BP component.

The relationship between the Cd-BP_{10} and Cd-BP_{20} is an important question to be resolved. If the Cd-BP_{20} are dimers of the Cd-BP_{10}, then why is dimer formation favored (under the experimental conditions identical to those employed in this study rat MTs never form dimers). If on the other hand the Cd-BP_{20} are in fact single polypeptide chains, then the origin and function of these proteins must be investigated.

In conclusion, it appears that there are Cd-BPs present in mollusks which differ significantly from mammalian

MTs. The relationship between the structure of these proteins and their function in these invertebrates will no doubt further our understanding of the biological role of metallothioneins.

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