

Mercury-Binding Proteins from the Marine Mussel, *Mytilus edulis*

by G. Roesijadi*

The marine mussel, *Mytilus edulis*, possesses low molecular weight, metal-binding proteins which can be induced by and, in turn, bind mercury when individuals are exposed to low, but elevated concentrations of mercury as HgCl_2 . Induction of the proteins by exposure of mussels to copper, cadmium, or mercury is associated with enhanced tolerance to mercury toxicity. Mercury-binding proteins isolated from gills of mussels occur as two molecular weight variants of about 20–25 and 10–12 kdaltons, respectively, on Sephadex G-75. These have been designated as HgBP_{20} and HgBP_{10} following the nomenclature used for cadmium-binding proteins. HgBP_{20} represents the primary mercury-binding species. These exist as dimers which can be dissociated into subunits by treatment with 1% 2-mercaptoethanol. Further purification of HgBP_{20} by DEAE-cellulose ion-exchange chromatography resulted in the resolution of three major mercury-binding protein peaks; analysis of two of these showed that both had similar amino acid compositions with 26% half-cystine, 16% glycine, and very low levels of the aromatic amino acids phenylalanine and tyrosine (0.3–0.5%), histidine (0.4%), methionine (about 0.5%), and leucine (about 1%). These are similar to the compositions of proteins reported as mussel thioneins by others. Separation of HgBP_{20} by anion-exchange high-performance liquid chromatography resulted in the resolution of six peaks, indicating a more complex situation than was evident from DEAE-cellulose separations. Although not completely purified, these also contain cysteine- and glycine-rich proteins.

Introduction

The marine mussel, *Mytilus edulis*, possesses low molecular weight, metal-binding proteins with general properties similar to those of metallothionein. Originally reported as cadmium-, copper-, and zinc-binding proteins (1), subsequent studies have shown that they also bind mercury (2) and can be induced by cadmium (3), copper (4), and mercury (5). Induction of these proteins by low levels of cadmium, copper, and mercury have been shown to be associated with enhanced tolerance to mercury toxicity (6,7), suggesting a protective or detoxification function against certain metal ions.

The results of studies on characterization of metal-binding proteins of *Mytilus edulis* have, thus far, been equivocal as to their identity and number of isomeric forms (2,3,8,9). Prior to this report, up to three isoforms, with one resembling metallothionein in amino acid composition, had been reported for cadmium-induced proteins (3), while a single protein identified as a metallothionein was reported for copper (9). An earlier report on mercury-binding proteins (2) described a protein similar in amino acid composition to that originally reported for the cadmium-binding protein in oysters (10), but unlike that

of metallothionein.

This study summarizes more recent work conducted on low molecular weight mercury-binding proteins of *Mytilus edulis*. The primary form of these proteins is that of a dimer of 20 to 25 kdaltons (kD) M_r on Sephadex G-75. These can be resolved into three charge variants following anion exchange chromatography on DEAE-cellulose and six following anion-exchange high-performance liquid chromatography. These findings, in conjunction with recent results reported in this volume which indicated four for both the 20 and 10 kD forms of mussel cadmium-binding proteins (11), indicate a highly complex situation for the metal-binding proteins of this organism.

Materials and Methods

Individuals of *Mytilus edulis* were collected from Sequim Bay, Washington, and exposed to 5 $\mu\text{g/L}$ mercury as HgCl_2 in a flowing seawater system described earlier (5). Gills were selected for study since mercury is primarily concentrated in this organ when mussels are exposed to mercury by the above mentioned.

Gills were excised and stored at -65°C . In later work, gills were placed in ice-cold homogenizing buffer, then frozen in buffer. Subsequent procedures were conducted on ice or at 4°C . Partially thawed gills (about 35–40 g wet weight) were homogenized in 20 mM Tris-HCl pH

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8.6 with a Tekmar SDT 100 EN probe. Homogenates were centrifuged at 80,000g for 90 min.

Supernatants were chromatographed on Sephadex G-75 (5 × 80 cm) equilibrated with 20 mM Tris-HCl, pH 8.6. Eluents were monitored for 254 or 280 absorbance (these proteins absorb strongly at either wavelength), and fractions were collected and analyzed for mercury as described previously (5). Those comprising the primary low molecular weight, mercury-binding proteins of 20 to 25 kD apparent M_r (2) were pooled, concentrated over Amicon YM2 ultrafiltration membranes, and frozen at -65°C. Peaks from four to five separations were thawed, pooled, rechromatographed on Sephadex G-75 (5 × 80 cm) to remove additional high molecular weight material from the mercury-binding proteins, then concentrated again as described above. The resultant sample served as the basis for subsequent procedures.

In order to verify the dimer structure of these proteins, an aliquot of the sample was first made up to 1% with 2-mercaptoethanol, incubated overnight, then washed and concentrated in an ultrafiltration cell (Amicon YM2 membrane). This step was followed by gel chromatography using Sephadex G-75 (1.6 × 80 cm) equilibrated with 20 mM Tris-HCl, pH 8.6, 10 mM 2-mercaptoethanol. The profile from this separation was compared to that of an aliquot not treated with 2-mercaptoethanol.

Anion-exchange chromatography on DEAE-cellulose (Whatman DE-52) used 15 g DE-52 (1.6 × approx. 15 cm column) initially equilibrated with 20 mM Tris-HCl, pH 7.4. An aliquot of the sample was adjusted to pH 7.4 with 50% HCl, applied to the column, washed with 20 mM Tris-HCl, pH 7.4, then eluted with a 500 mL linear gradient of 20 to 300 mM Tris-HCl, pH 7.4. Eluents were monitored for UV absorbance and fractions were analyzed for mercury as above. For separation of samples which had previously been reduced with 2-mercaptoethanol, all solutions also included 10 mM of this reagent. A modified gradient (see Results section) was also used in larger scale separations in which all of the sample from the initial Sephadex separations was applied to the DE-52 column.

Anion-exchange high-performance liquid chromatography (AX-HPLC) utilized an AX-300 column (4.6 × 25 cm, Brownlee Laboratories) equilibrated in 20 mM Tris-HCl, pH 8, at room temperature, with a flow rate of 2.3 mL/min. Proteins were eluted with a 30 min gradient of 0 to 300 mM NaCl in 20 mM Tris-HCl, pH 8. This pH will reduce column life of silica gel-based columns, but was found in preliminary experiments to achieve the best peak separations. The sample pH was not adjusted in this case since the pH of Tris in the sample was close to 8 at room temperature. The chromatographic system consisted of Waters dual Model 6000 pumps, 580 gradient programmer, and WISP sample processor. Absorbance was monitored at 254 nm, and fractions were analyzed for mercury.

The amino acid composition of proteins was determined by following performic acid oxidation and hydrolysis for 24 hr in 6 N HCl.

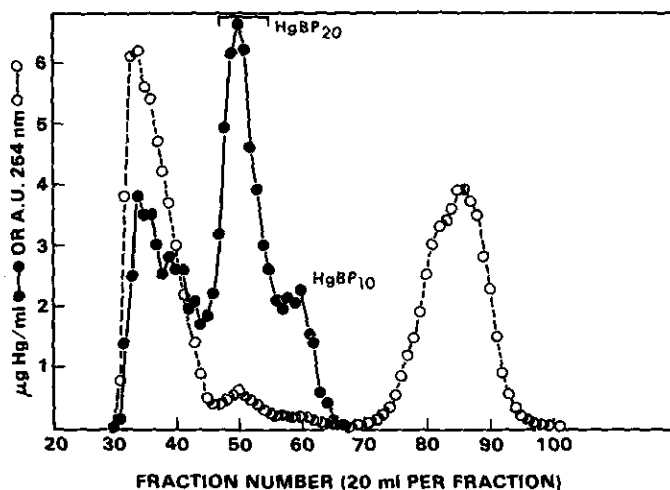


FIGURE 1. Gel chromatography of soluble fraction of *Mytilus edulis* on Sephadex G-75 (5 × 80 cm).

Results and Discussion

Mercury-binding proteins from the gills of *Mytilus edulis* occur as two molecular weight variants with M_r of 20 to 25 and 10 to 12 kD on Sephadex G-75 (Fig. 1). These have been designated as HgBP₂₀ and HgBP₁₀, respectively, following the terminology of Frazier (11). The former is the primary low molecular weight mercury-binding species in the mussel. Treatment of HgBP₂₀ with 1% 2-mercaptoethanol causes a shift in molecular weight to about half the original value (Fig 2). It is likely, then, that HgBP₂₀ is a dimer with subunits of about 10 kD. The apparent mechanism of dimerization of mercury-binding proteins is through disulfide bridges, a hypothesis consistent with earlier findings (12).

Further separation of the HgBP₂₀ fraction on DEAE-cellulose resulted in the resolution of three peaks eluting between 100 and 200 mM Tris at pH 7.4 (Fig. 3). Separation of the HgBP₂₀ fraction on DEAE-cellulose after

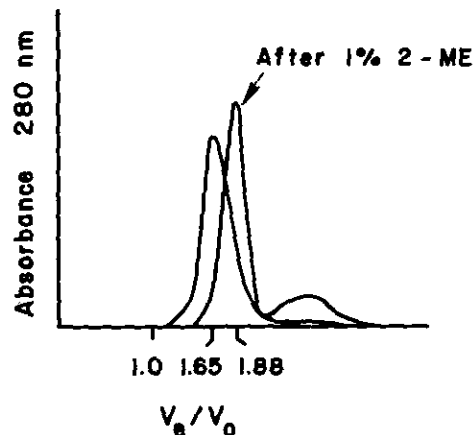


FIGURE 2. Gel chromatography of HgBP₂₀ before and after reduction with 1% 2-mercaptoethanol. Elution buffer contained 10 mM 2-mercaptoethanol.

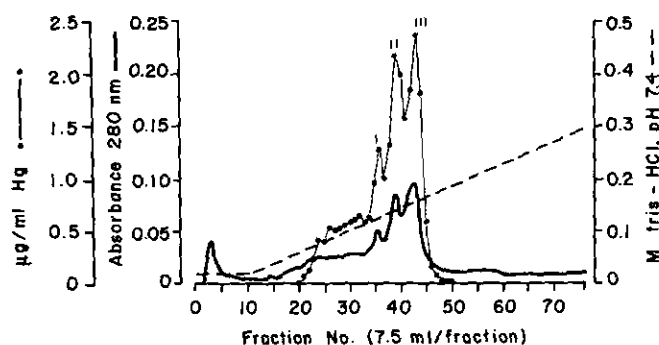


FIGURE 3. Anion exchange chromatography of HgBP₂₀ on DEAE-cellulose (Whatman DE-52, 1.6 × 15 cm).

reduction with 2-mercaptoethanol and dissociation into subunits resulted in a similar profile (Fig. 4). The similarities in elution profiles of the dimer fraction obtained before and after dissociation into subunits indicated that the subunits possessed net charges not unlike those of the dimer.

A larger-scale purification of the unreduced HgBP₂₀ fraction on DEAE-cellulose also resulted in the resolution of three major peaks corresponding to those above, but with considerably more overlap. Therefore, the fractions containing the three peaks were pooled, adjusted to initial buffer conditions, and rerun on DEAE-cellulose using a modified gradient, as shown in Figure 5. The three peaks were more cleanly separated from each other on this run. However, a fraction collector malfunction resulted in loss of the first of the three peaks. The last two were collected, concentrated by ultrafiltration (Amicon YM-2 membrane), exchanged to 50 mM NH₄HCO₃ by gel chromatography on Sephadex G-75, and lyophilized. Amino acid analysis of the protein preparations obtained showed generally similar compositions (Table 1). Both were rich in half-cystine (26%) and glycine (16%) and very low in the aromatic residues phenylalanine and tyrosine (0.3–0.5%), histidine (0.4%), methionine (about 0.5%), and leucine (about 1%). These values are similar to those of the half-cystine rich cadmium-binding proteins (8,11).

When the HgBP₂₀ fraction obtained from Sephadex G-75 (Fig. 1) was separated by AX-HPLC, six mercury-binding protein peaks eluted between 18 and 28 min.

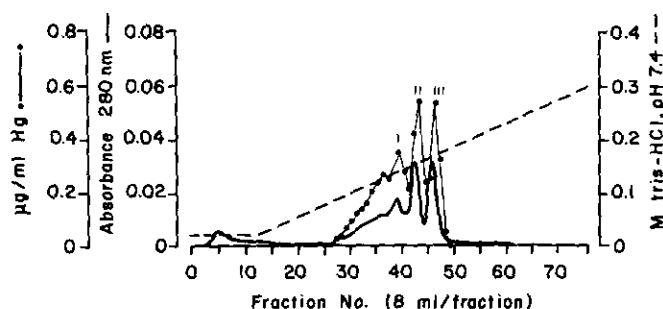


FIGURE 4. Anion exchange chromatography of HgBP₂₀ on DEAE-cellulose (Whatman DE-52, 1.6 × 15 cm). Elution buffer contained 10 mM 2-mercaptoethanol.

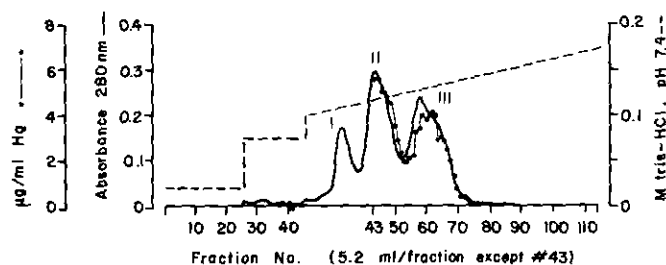


FIGURE 5. Anion exchange chromatography of HgBP₂₀ on DEAE-cellulose (Whatman DE-52, 1.6 × 20 cm) with modified gradient as shown. The sample was initially chromatographed using conditions in Fig. 3.

(Fig. 6). These results demonstrate greater complexity than is evident from DEAE-cellulose separations. Preliminary amino acid analysis of these six peaks showed high, but variable, amounts of half-cystine (9–21%) and glycine (12–14%). Increased levels of aromatic amino acids and minor residues were measured in these preparations as well. These were negatively correlated with the amounts of half-cystine on the different peaks, and it appears that removal of components which contain these latter residues may enable purification of proteins with higher half-cystine and glycine content from these peaks. Current work is focused on additional purification of these HPLC preparations.

Results to date indicate that the low molecular weight, mercury-binding proteins of *Mytilus edulis* are very similar to proteins induced by cadmium (3,8,11) and copper (9). Common properties include the presence of dimer and monomer forms following mild extraction conditions; multiple forms of the dimer; high half-cystine and glycine content; and the absence of or very low levels of phenylalanine, tyrosine, histidine, leucine, and methionine. The lack of methionine is consistent with the reported composition of some other nonmammalian (13–15), but not mammalian, metallothioneins. In the latter, *N*-acetylmethion-

Table 1. Amino acid composition of *Mytilus edulis* mercury-binding proteins obtained after purification of HgBP₂₀ by DEAE-cellulose anion-exchange chromatography (residues per cent).

Amino acid	Composition, residues - %	
	II	III
1/2cys	26.3	25.7
Asx	9.2	10.2
Thr	7.1	7.2
Ser	9.4	7.5
Glx	4.2	4.3
Pro	4.1	4.0
Gly	16.7	16.2
Ala	4.4	3.8
Val	3.9	4.8
Met	0.3	0.6
Ile	2.8	3.0
Leu	0.9	1.3
Tyr	0.3	0.6
Phe	0.3	0.5
His	0.4	0.5
Lys	7.9	7.5
Arg	1.8	2.6

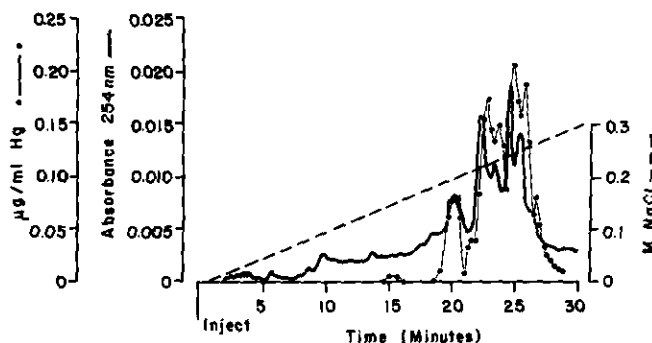


FIGURE 6. Anion-exchange high-performance liquid chromatography (4.6×25 cm AX-300 column, Brownlee Laboratories) of HgBP₂₀. Column equilibrated in 20 mM Tris-HCl pH 8.0.

ine represents the amino terminus (16).

Of direct relevance to the present work is a recent study on cadmium-binding proteins of *Mytilus edulis* which has indicated four forms each for CdBP₂₀ and CdBP₁₀ (11). Up to six have been shown here for HgBP₂₀, the primary low molecular weight mercury-binding proteins. HgBP₁₀ is yet to be studied in detail, but will contribute at least one more component. The significance of these large numbers of multiple forms is not known. Recognition of their presence adds an additional challenge to understanding the chemical nature and function of these proteins.

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