HISTIDINE-RICH GLYCOPROTEIN FROM THE HEMOLYMPH OF THE MARINE MUSSEL MYTILUS EDULIS L. BINDS CLASS A, CLASS B, AND BORDERLINE METALS

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(Rceived 3 July 2006; Accepted 21 November 2006)

Abstract—Few studies have directly addressed the question of how metals (both essential and nonessential) are transported in the circulatory system of bivalve mollusks. One potential metal-transport protein, histidine-rich glycoprotein (HRG), has previously been isolated and characterized from the blood plasma of the marine mussel Mytilus edulis L. The present study was undertaken to investigate the extent to which mussel HRG can bind a variety of essential and nonessential metals in vitro, using immobilized metal-ion affinity chromatography (IMAC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The equilibrium metal speciation model MINTEQ2 was used to compute the amount of metal that bound to the IMAC packing material during the charging and initial wash steps. Results demonstrated that HRG can bind all seven of the metals tested (Ca, Cd, Hg, Mg, Ni, Pd, and Zn) and that HRG is the only metal-binding protein in IMAC eluents. Because HRG-metal binding strengths (log $K_a$) likely correspond with histidine–metal binding strengths, and because HRG is the predominant mussel plasma protein, the majority of each of the seven metals probably would be present in mussel blood as protein-bound metal rather than as free metal ion. The finding that a single mussel plasma protein may be responsible for binding all these metals raises important questions about how these different metals are subsequently transferred from HRG to different tissues of the mussel, where they may exhibit tissue-specific patterns of utilization, sequestration, elimination, and toxicity.

Keywords—Histidine-rich glycoprotein Immobilized metal-ion affinity chromatography Metal binding Metal transport Mussel

INTRODUCTION

Bivalves play important roles in the ecology of complex marine communities, many of which have suffered disturbances from a number of anthropogenic activities, including contamination by metals and other persistent chemicals. Because of their importance to the community as well as their relatively high resistance and low metabolism of contaminants cause of their importance to the community as well as their contamination by metals and other persistent chemicals. Bivalve communities, many of which have suffered disturbances from anthropogenic activities, including contamination by metals and other persistent chemicals. The goal of the present study was to further investigate the extent to which HRG from the blood plasma of the marine mussel $M$. edulis. Those authors reported that HRG constitutes approximately 60% (w/w) of the total plasma proteins in the mussel blood, thus suggesting a dominant role in circulatory transport processes. They also determined that this glycoprotein contains almost 14% (w/w) histidine and 12% (w/w) carbohydrate (primarily hexose), exhibits high-affinity Cd-binding sites (log $K_a$ = 7.65) and 10 low-affinity sites (log $K_a$ = 5.41) [18]. Mussel HRG appears to be involved in the rapid transfer of Cd from the blood plasma to the mussel kidney [19], thereby demonstrating a potentially important role in metal transport and transfer in this species. The goal of the present study was to further investigate the extent to which HRG from the blood plasma of $M$. edulis can bind a wide variety of both essential and nonessential metals. Widespread binding of a broad array of metals would support the hypothesis that HRG functions as a major metal-transport protein in mussel blood.

MATERIALS AND METHODS

Organisms and extraction of plasma

*Mussel Mytilus edulis* L. (length, 4–7 cm) was collected from the low intertidal zone in Boston Harbor off Columbia Point...
Plasma was decanted, frozen in liquid nitrogen, and stored at equilibration with 50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer containing 1 M NaCl (pH 7.5; ~30 ml). Cell-free M. edulis blood plasma (2–3 ml containing 1.3–4.0 mg of protein) was introduced onto a metal-IDA column (length, 15 cm; width, 15 cm; height, 0.15 cm; stacked at 50 mA; resolved at 100 mA) at 15°C. Protein bands were stained with Coomassie brilliant blue R-250 and their migration distances compared to molecular weight standards (lysozyme, 14.44 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa; and phosphorylase, 97.4 kDa).

**Metal speciation modeling**

The equilibrium metal speciation model MINTEQA2 version 4.02 [23] was used to estimate the amount of each metal bound to the IDA packing material following the charging and initial wash steps. The CA2+ and Pb2+ were added to the MINTEQA2 components list (file Comp.dbs). Metal-IDA and metal-IDA2 stability constants (K) for Ca, Mg, Cd, Ni, Zn, Hg, and Pd, as well as stability constants for Pb(OH)2, Pb(OH)3, PbCl2, PbCl3, CdCl2, CdCl3, CaCl2, Ca(OH)2, MgCl2, and Mg(OH)2, were obtained from the National Institute of Standards and Technology Standard Database 46 version 5.0 [24], corrected (when necessary) to 0.0 M ionic strength using the Davies equation and to 25°C (when standard enthalpy values were available) using the van’t Hoff equation, and added to the MINTEQA2 thermodynamic database (file Thermo.dbs). Because a Hg-IDA2 stability constant was not available, an estimate of the value was obtained from the regression of metal-IDA2 log K versus metal-IDA log K for the remaining six metal pairs (y = 1.47x + 1.03, r2 = 0.992). Based on model estimates for the speciation of each metal, the amount of protein recovered in the EDTA elution was normalized to the estimated metal content of the column (µg protein/µmole metal) as well as to the amount of IDA packing material (µg protein/g IDA).

**Statistical analysis**

All statistical analyses were run on SPSS version 12.0 for Windows, with p ≤ 0.05 accepted as the level of significant differences. The relationship between the amount of plasma protein added to each of the metal IMAC columns and the amount of protein subsequently eluted off the column with EDTA was assessed using two-tailed Pearson bivariate correlation. Differences in the amount of protein obtained for each of the seven metal IMAC treatments (µg protein/µmole metal and µg protein/g IDA) were compared using one-way analysis of variance (ANOVA), followed by a Duncan a posteriori contrast. Raw data were log transformed before ANOVA to comply with the criterion for homogeneity of variances.

**RESULTS**

All seven of the metals examined (two Class A, two Class B, and three borderline metals) produced an IMAC elution pattern similar to that reported by Nair and Robinson [17] for Cd, even though the amount of IDA used was scaled down from that employed in the original study (Fig. 1, with Mg, Hg, and Zn presented as examples). Two protein peaks that absorbed strongly at 280 nm were resolved. The first broad peak contained nonbound plasma protein as well as excess HRG that washed through the column soon after the sample was introduced. Lengthy washing of the column with buffer yielded no additional peaks. A 50 mM EDTA elution yielded the second peak. Elution peak heights were variable from run to run, but Zn typically exhibited a large peak and the Class A (Ca and Mg) and Class B (Hg and Pd) metals relatively small elution peaks.

As expected for an IMAC procedure designed with a limited number of metal-binding sites, the amount of protein eluted...
with EDTA showed no relationship to the amount of protein added to the column, either for the individual metal preparations or within the entire database (data from all metals combined; Pearson correlation, \( p > 0.05 \)). Because HRG makes up approximately 60% (w/w) of the plasma proteins [17], much of the HRG in each sample washed through the column. The amount of protein eluted with EDTA from the Ca-, Mg-, Cd-, Ni-, and Hg-IMAC columns ranged from 28 to 219 \( \mu \)g (2–11% [w/w] of the protein loaded onto the column). Protein concentrations could not be determined for Pd, because Pd compounds interfered with the Bradford protein assay and appreciably contributed to absorbance readings at 280 nm. The Zn protein elutions were the most variable of all the metals, ranging from 169 to 826 \( \mu \)g (7–27% [w/w] of the protein loaded).

The protein eluted with EDTA from each of the replicates of all seven metal-IMAC preparations was identified as HRG using SDS-PAGE (Table 1) based on a polymorphic molecular weight distribution similar to that reported by Nair and Robinson [17]. One, two, or three bands typically were seen on the gel, with molecular weights ranging from 32 to 39 kDa, respectively (Table 1), and more bands typically observed when more protein was obtained in the elution peak and loaded onto the gel. The slight variability (1–2 kDa) observed in the molecular weights calculated for these bands among the seven metal preparations resulted from slight variations in resolving molecular weights of proteins \( \leq 40 \) kDa on 7.5% SDS-PAGE gels. Cell-free *Mytilus edulis* blood plasma, which was not subjected to IMAC, displayed similar microheterogeneity in this molecular weight range. It is likely, for example, that the 32-, 36-, and 38-kDa bands observed for the Ni- and Zn-IMAC preparations correspond to the 35-, 37-, and 39-kDa bands reported by Nair and Robinson [17]. The staining intensity of the HRG bands varied among metal preparations but appeared to directly correlate with protein loading (Table 1). No bands other than HRG were observed on the gels, indicating that metal-binding proteins with affinity constants for the tested metals appreciably higher than those for HRG were not present in *M. edulis* blood.

![Fig. 1. Chromatogram from immobilized metal-ion affinity chromatographic separations of Mytilus edulis blood plasma, using a Class A– (Mg; – –), borderline- (Zn; – –), and Class B– (Hg; – –) charged iminodiacetate (IDA) column. Columns (length, 40; inner diameter, 8 mm) were loaded with an estimated 17.1 \( \mu \)mole Mg/g, 4.8 \( \mu \)mole Hg/g, and 20.5 \( \mu \)mole Zn/g of IDA-Sepharose. Plasma samples (2–3 mg of total protein) were loaded and then washed with 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (pH 7.5) containing 1 M NaCl, and metal-bound protein was eluted with 50 mM ethylenediaminetetraacetic acid. Fraction number represents 1-ml samples.](Image 45x585 to 279x738)

![Fig. 2. Metal-binding to the iminodiacetate (IDA)–Sepharose 6B packing material (\( \mu \)mole metal/g IDA packing material) as estimated by MINTEQA2 version 4.02 [16]. Dark bars represent metal loading following metal charging; white bars represent metal remaining after subsequent washing with equilibration buffer (50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.5, with 1 M NaCl). The dashed horizontal line (26 \( \mu \)mole/g) represents the midpoint of the manufacturer’s reported Zn-binding capacity (22–30 \( \mu \)mole/ml).](Image 306x120 to 546x296)

### Table 1. Molecular weights and staining intensity of proteins eluted from immobilized metal-ion affinity chromatographic (IMAC) iminodiacetate columns with 50 mM ethylenediaminetetraacetic acid (EDTA) subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue R250.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Molecular weight of HRG bands (kDa)</th>
<th>Amount of protein loaded (( \mu )g)</th>
<th>HRG staining intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class A metals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>35, 39</td>
<td>40</td>
<td>++</td>
</tr>
<tr>
<td>Mg</td>
<td>36, 39</td>
<td>100</td>
<td>++</td>
</tr>
<tr>
<td>Borderline metals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cd</td>
<td>39</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>Ni</td>
<td>32, 36, 38</td>
<td>100</td>
<td>+++</td>
</tr>
<tr>
<td>Zn</td>
<td>32, 36, 38</td>
<td>100</td>
<td>+++</td>
</tr>
<tr>
<td>Class B metals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hg</td>
<td>36, 39</td>
<td>50</td>
<td>++</td>
</tr>
<tr>
<td>Pd</td>
<td>38</td>
<td>NM</td>
<td>+</td>
</tr>
</tbody>
</table>

*Data are typical examples from one of the replicate SDS-PAGE analyses for each metal-IMAC treatment. HRG = histidine-rich glycoprotein; NM = not measurable because of interference from Pd with the Bradford protein assay.*

Each of the seven metals did not bind to the IDA packing material to the same extent (Fig. 2). The MINTEQA2 modeling of Zn-IDA binding confirmed the reported Zn-binding capacity of the packing material (24.6 mmol/g vs the manufacturer’s estimate of 22–30 mmol/ml). In general, metal-IDA affinities were low for Class A, intermediate for borderline, and high for Class B metals (Table 2). We therefore expected that IDA would bind more Class B than Class A metals (i.e., Class B > borderline > Class A) and that HRG recoveries would follow this same sequence. The MINTEQA2 modeling demonstrated that this pattern generally held for Class A and borderline metals. Class B metals, however, were anomalous, primarily because of their strong complexation to various chloride species. Mercury exhibited stronger binding to chlorides than...
Table 2. Stability constants ($\log K_s$) for seven metals and iminodiacetate (IDA)*

<table>
<thead>
<tr>
<th>Class</th>
<th>Complex</th>
<th>$\log K_s$</th>
<th>Complex</th>
<th>$\log K_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Ca-IDA</td>
<td>3.37*</td>
<td>Ca-histidine</td>
<td>1.64</td>
</tr>
<tr>
<td>A</td>
<td>Ca-IDA₂</td>
<td>5.9*</td>
<td>Ca-histidine₂</td>
<td>—</td>
</tr>
<tr>
<td>A</td>
<td>Mg-IDA</td>
<td>3.8*</td>
<td>Mg-histidine</td>
<td>—</td>
</tr>
<tr>
<td>A</td>
<td>Mg-IDA₂</td>
<td>5.71</td>
<td>Mg-histidine₂</td>
<td>—</td>
</tr>
<tr>
<td>Borderline</td>
<td>Cd-IDA</td>
<td>6.57</td>
<td>Cd-histidine</td>
<td>6.09</td>
</tr>
<tr>
<td>Borderline</td>
<td>Cd-IDA₂</td>
<td>10.96</td>
<td>Cd-histidine₂</td>
<td>10.58</td>
</tr>
<tr>
<td>Borderline</td>
<td>Ni-IDA</td>
<td>9.24*</td>
<td>Ni-histidine</td>
<td>9.09</td>
</tr>
<tr>
<td>Borderline</td>
<td>Ni-IDA₂</td>
<td>15.7*</td>
<td>Ni-histidine₂</td>
<td>16.16</td>
</tr>
<tr>
<td>Borderline</td>
<td>Zn-IDA</td>
<td>8.01</td>
<td>Zn-histidine</td>
<td>6.94</td>
</tr>
<tr>
<td>Borderline</td>
<td>Zn-IDA₂</td>
<td>13.26</td>
<td>Zn-histidine₂</td>
<td>12.68</td>
</tr>
<tr>
<td>B</td>
<td>Hg-IDA</td>
<td>12.66</td>
<td>Hg-histidine</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Hg-IDA₂</td>
<td>19.6*</td>
<td>Hg-histidine₂</td>
<td>21.94*</td>
</tr>
<tr>
<td>B</td>
<td>Pd-IDA</td>
<td>18.32</td>
<td>Pd-histidine</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>Pd-IDA₂</td>
<td>27.62</td>
<td>Pd-histidine₂</td>
<td>—</td>
</tr>
</tbody>
</table>

* Values, obtained primarily from the National Institute of Standards and Technology Database 46 version 5.0 [17], were either used as reported (*) or corrected to 25°C and 0.0 M ionic strength.

Brooks and Davidson [31].

Pd exhibited. Because the Hg-IDA column was charged with HgCl₂, much of the Hg remained as chloride complexes (~75% [w/w]) rather than as IDA complexes (~25% [w/w]), even during the initial metal-charging step. Whereas HEPES buffer itself binds virtually no metal [25], the chloride included in the 1 M NaCl wash buffer (used to prevent HRG denaturation) stripped off more than 50% (w/w) of the Hg that initially bound to the IDA column. The impact of chloride complexation also was observed for all the other metals tested, but to a much lesser degree than for Hg and Pd. For example, Class A metals (Ca and Mg) showed relatively weak chloride binding, but other anions, such as hydroxides, were relatively more important inorganic species. Following the wash step, the borderline metals Ni and Zn were retained by the IDA to a greater extent than the other metals tested, whereas the Class B metal Hg was retained the least (Fig. 2).

The greatest amount of HRG was recovered from IMAC IDA columns charged with Zn (one-way ANOVA, $p < 0.05$) (Fig. 3A). As noted, results for Zn were quite variable, however, with recoveries of between 71 and 351 μg HRG/g IDA ($n = 4$ Zn preparations). Nevertheless, even if the highest HRG recovery value was considered to be anomalous and eliminated from the statistical analysis, Zn-IDA still retained a higher amount of HRG compared to the other metals (one-way ANOVA, $p < 0.05$; $n = 3$ Zn preparations).

The much higher HRG recoveries from the Zn-charged IDA columns compared with the Ni-charged columns (Fig. 3) was not expected based on the metal-loading estimates shown in Figure 2 and the relatively strong affinity of both Zn and Ni for histidine groups (Table 2). The IDA columns retained high amounts of both Zn and Ni, providing ample binding sites for HRG. It might be expected that Ni therefore would show equal- or stronger HRG recoveries. Because Ni-histidine and Ni-histidine₂ affinity constants, however, were similar to the Ni-IDA and Ni-IDA₂ affinity constants (Table 2), HRG was very effective in competing with IDA for Ni, resulting in a loss of Ni from the IDA column during plasma passage. Because Zn-histidine affinity constants were appreciably lower than Zn-IDA stability complexes (Table 2), HRG did not disrupt Zn-IDA complexes as plasma traveled through the column.

DISCUSSION

The MINTEQA2 thermodynamic equilibrium modeling was an important component of the present study. It helped to explain why different HRG recoveries were obtained with different metals, and it demonstrated that the interactions among HRG, IDA, metals, and other inorganic components of the immediate environment were highly complex. A cursory examination of metal-binding constants would have provided little insight regarding the outcome of the IMAC experiments. The MINTEQA2 modeling allowed us to examine the myriad of inorganic speciation reactions (e.g., chlorides and hydroxides) that developed during the various stages of the IMAC procedure and influenced the
final recovery of HRG (e.g., the unexpected differences in protein recovery in the Ni-IMAC vs the Zn-IMAC experiments and the low charging of the IDA columns with Hg). Although clearly useful for understanding metal interactions that occurred during the present experiments and in our previous work in vivo [19,26], MINTEQ2 modeling is constricted, however, by the limited availability of high-quality metal stability constants. We could not locate critical stability constants for a number of histidine–metal species, and experimentally derived mussel metal-HRG stability constants were only available for Cd [18]. Because the reported log $K_4$ for Cd-HRG (7.65 for six high-affinity HRG sites) [18] lies between the reported log $K_4$ values for Cd-histidine (6.09) and Cd-histidine$_2$ (10.58) [24], we can reasonably expect that the log $K_4$ values for the other HRG–metal complexes would be similar to those for the histidine–metal complexes, but we have not as yet attempted to use these values in our MINTEQ2 modeling. Finally, although we obtained stability constants for the predominant metal species from a source with rigorous quality control [24], a number of low-affinity species were not included, because the quality of the data could not be assured. These low-affinity species, if present at high enough concentrations, could be important. Nevertheless, the coupling of metal speciation modeling with direct experimentation, such as the approach used in the present study, will, in future studies, lead to a better understanding of such important processes as metal uptake [27] and blood-borne metal transport [19,26].

We have demonstrated that *M. edulis* HRG binds a wide variety of metals, spanning the entire range from Class A (Ca and Mg) through borderline (Cd, Ni, and Zn) to Class B (Hg and Pd) metals. Not only is HRG the predominant mussel plasma protein (~60% [w/w] of total plasma proteins) [17], it is the sole plasma protein that was isolated by IMAC using the seven metals examined in the present study. Because IMAC separated HRG from unmodified mussel plasma (i.e., no disturbance of the inorganic or organic matrix of the samples), HRG likely is able to bind each of the seven metals in vivo. Although circumstantial, this evidence supports the hypothesis that mussel HRG is a metal-transport protein that binds and circulates a variety of both essential metals (e.g., Ca, Mg, Ni, and Zn) and nonessential metals (e.g., Cd, Hg, and Pd) in the circulatory system of *M. edulis*.

Our results augment the earlier findings by Renwrantz et al. [15], who described a 35-kDa protein (SPB1) in *M. edulis* from the eastern Atlantic Ocean, although their serum protein did not exhibit microheterogeneity with SDS under reduced conditions. The SPB1 bound to organomercural agarose and was precipitated with cadmium and lead acetate, CuCl$_2$, HgCl$_2$, and ZnCl$_2$. As a result, those investigators proposed that SPB1 is involved in metal transport. Later, Hattan et al. [28] isolated a 28-kDa glycoprotein (extrapallial glycoprotein [EP]; mol wt determined by time-of-flight mass spectrometry rather than by SDS-PAGE) from the extrapallial fluid of *M. edulis*. This protein was histidine-rich (11%), bound Ca, exhibited microheterogeneity (mass spectroscopic peaks at 27, 27.8, 28.3, and 29 kDa), and comprised 56% of the total protein in the extrapallial fluid. Higher-molecular-weight bands could be produced by adding Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, or Cd$^{2+}$ to EP samples before native PAGE, indicating that EP was able to bind these metals [28]. Electron paramagnetic resonance studies have recently shown that EP also binds Cu$^{2+}$ [29]. Because the secondary structure of EP was reversibly altered on binding Ca, EP was hypothesized to be involved in shell deposition [28].

It is likely that SPB1, EP, and HRG are identical. The SPB1 protein shows functional similarities to the other two proteins, but to our knowledge, the amino acid sequence has not been reported. Recently, Yin et al. [29] published the cDNA-derived protein sequence of EP (GenBank accession no. AY364453). This 213-amino-acid sequence exhibited 95.8% similarity with the sequence of a heavy metal (Cd, Cu, and Zn)–binding protein, HIP [30], which is a plasma protein obtained from gill, mantle, and digestive gland tissue of *M. edulis*. The EP glycoprotein shows a 96.5% similarity with the incomplete (198-amino-acid), cDNA-derived protein sequence that we have determined for HRG (M. Gaudette and W.E. Robinson, Department of Environmental, Earth, and Ocean Sciences, University of Massachusetts–Boston, Boston, MA, USA, unpublished data). There seems to be little doubt, therefore, based on the amino acid sequence and functional similarities, that EP, HIP, and HRG are identical proteins. The present study demonstrates that this protein’s metal-binding properties are not limited to a single Class A metal (e.g., Ca) or to a group of metals from one or two classes but, rather, cover the entire range of Class A, Class B, and borderline metals.

Because of the ability of HRG to bind a wide range of metals, and because HRG is the predominant protein in the blood of *M. edulis* [17], very little metal likely is present in mussel blood as free metal ion. For example, more than 93% of the Cd injected into *M. edulis* blood was chelated by HRG, leaving a very small percentage as inorganic species and less than 2% as the Cd$^{2+}$ free metal ion [19]. Similarly, only 2.4% of all the calcium normally present in the blood of *Mercenaria mercenaria* was present as Ca$^{2+}$; the rest was primarily bound to a plasma protein or to proteins with a molecular weight larger than 1,000 Da [26]. These results raise the possibility that free metal ions are not the predominant species of metal that are transferred from the blood, cross membranes, and enter various tissues. Because the bulk of metals are expected to be bound to HRG, we predict that HRG is intricately involved not only in metal transport in blood but also in this transfer process.

This possibility raises intriguing questions. Just how are metals differentially transferred to various tissues if they are all bound to the same transport protein? We have experimental evidence to indicate that HRG appears to be involved in a rapid mechanism for delivering Cd to the kidney of mussels, yet this rapid mechanism is not exhibited by other tissues of the organism [19]. Will HRG deliver other metals to the kidney as well? How does HRG pass off metals to tissues that exhibit differences in their abilities to take up, sequester, detoxify, or eliminate various metals? Does a metal-HRG complex cross membranes, or is the metal passed off to receptors with higher metal affinities? Our current work focuses on this interface between the blood transport and the transfer of Cd to bivalve kidney cells. In a broader sense, however, our questions point out that a great deal of additional work is clearly needed to more fully determine how both essential and nonessential metals are transferred from plasma proteins to critical sites in bivalve tissues.

Acknowledgement—The present research was supported by grant 5710001173 from the National Sea Grant Office, Environmental Marine Biotechnology Program, Washington, DC.

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Mussel HRG binds A, B, and borderline metals


