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Identification and quantification of histidine-rich glycoprotein (HRG) in the blood plasma of six marine bivalves

Adal T. Abebe [a], Samantha J. Devoid [a], Manickam Sugumaran [a], Ron Etter [a], William E. Robinson [b,*]

[a] *Department of Biology, University of Massachusetts Boston, Boston, MA 02125-3393, USA*
[b] *Department of Environmental, Earth and Ocean Sciences (EEOS), University of Massachusetts Boston, 100 Morrissey Blvd., Boston, MA 02125-3393, USA*

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Abstract

Histidine-rich Glycoprotein (HRG) is a metal-binding protein described from the blood plasma of a pteriomorph bivalve, the marine mussel *Mytilus edulis* L. We demonstrate here, using Cd-Immobilized Metal Affinity Chromatography (IMAC), SDS-PAGE, Western Blotting, and ELISA, that HRG is present in three additional pteriomorphs and two heterodont bivalves. ELISA indicates that HRG is the predominant blood plasma protein in all six species (41 to 61% of total plasma proteins by weight). Thus, HRG appears to be a widespread metal-binding protein in the plasma of bivalves.

Keywords: Bivalve; Blood plasma; Cadmium; ELISA; HRG; IMAC; Metal carrier; Transport protein

1. Introduction

Blood-borne metal transport proteins (e.g. serum albumin, ceruloplasmin, transferrin, histidine-rich glycoprotein) have been well-characterized in vertebrates, but have been little-studied in the invertebrates. Serum albumin, for example, is both the most abundant plasma protein and the primary metal-transport protein in mammals (Scott and Bradwell, 1983; Sarkar, 1989; Trisak et al., 1990), but is absent in non-vertebrate species (Peters, 1975; Metcalf et al., 2003). Invertebrate ceruloplasmins have yet to be identified, although transferrins have been isolated from the hemolymph of the Dungeness crab, *Cancer magister*, (Huebers et al., 1982) and several insect species (Winzerling and Law, 1997). Histidine-rich glycoprotein (HRG) is a metal-binding α_2-macroglobulin first isolated from human plasma (Heimburger et al., 1972), and further identified and characterized in a variety of mammalian species (Sørensen et al., 1993 and included cites). Mammalian HRG is a member of the cystatin superfamily of proteins (Koide and Odani, 1987) that constitutes less than 1% of the total plasma proteins (Scott and Bradwell, 1983). It is known to bind to different components of the coagulation and fibrinolytic systems (heparin, plasminogen and fibrinogen; Lijnen et al., 1983; Leung et al., 1984), as well as platelets (Lerch et al., 1988), heme (Burch and Morgan, 1985), and the transition metals Cd, Co, Cu, Hg, Ni and Zn (Morgan, 1981; Gluthans and Morgan, 1982), yet its primary function has yet to be determined.

Interest in invertebrate metal transport proteins has steadily increased over the past decade. In our own lab, for example, a polymorphic Cd-binding protein was noted in *Mytilus edulis* L. blood plasma (Robinson et al., 1997) and subsequently isolated, characterized and identified as a "histidine-rich glycoprotein (HRG)" (Nair and Robinson, 1999). The isolated protein contained 14% histidine, 21% Asx and Glx residues, and 12% carbohydrate (primarily hexoses; Nair and Robinson, 1999). In addition to the three principal glycoprotein bands observed on SDS-PAGE (35, 37 and 39 kDa), a weakly staining non-glycosylated band (29 kDa) was observed in some gels. Ferguson plots (from native PAGE gels) indicated an M_r of 63 kDa for the native protein. It was suggested that the 63 kDa protein was a dimer of the 39, 37, 35 and 29 kDa monomers.

* Corresponding author. Tel.: +1 617 287 7456; fax: +1 617 287 7474.
E-mail address: william.robinson@umb.edu (W.E. Robinson).

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HRG comprised ~60% of the plasma proteins (Nair and Robinson, 1999), bound Cd relatively strongly (6 high affinity binding sites, log K_a=7.6; 10 low affinity sites, log K_a=5.4; Nair and Robinson, 2001a), complexed a number of other metal ions (Ca, Hg, Mg, Ni, Pd and Zn; Devoid et al., in press), and participated in the transfer of Cd from mussel blood plasma to the kidney (Nair and Robinson, 2001b).

Over this period of time, it became apparent that HRG shared similarities with three other proteins that have been identified from *M. edulis*: SPB1 (Renwrantz et al., 1998); EP protein (Hattan et al., 2001; Yin et al., 2005); and HIP (Schneeweiss et al., 2002). SPB1 (serum protein band #1) was separated from mussel hemolymph samples using both native and SDS-PAGE, and characterized as a dominant blood protein with an M_r of 35± 1.8 kDa that interacted strongly with Cd, Cu, Hg, Pb and Zn (Renwrantz et al., 1998). The SPB1 protein band was quite thick when concentrated solutions were run on PAGE gels, suggesting possible microheterogeneity. EP protein (extrapallial protein) was isolated from the extrapallial fluid of *M. edulis* (Hattan et al., 2001). It was characterized as a 56.7 kDa dimer composed of two microheterogeneous monomers of either 27, 27.8, 28.3 and 29.0 kDa. plus an ~4 kDa N-linked glycan composed of hexose sugars (14% carbohydrate; Hattan et al., 2001). Similar to HRG, EP protein was found to contain 11% histidine, 25% Asx and Glx amino acids, and made up 56% of the protein in the extrapallial fluid. The addition of relatively high concentrations of Ca, Cd, Mg, and Mn to purified EP protein resulted in further aggregation of the homodimer, as shown by the appearance of several, very slowly migrating higher M_r bands in native gels (Hattan et al., 2001).

Recent work has further characterized EP protein (Yin et al., 2005). N-deglycosylated EP monomers were still able to form dimers, and continued to exhibit multiple bands on 2-dimensional PAGE gels (although at ~4 kDa lower M_r due to the removal of the glycan). This indicated that the protein's microheterogeneity was not primarily due to differences in the size of the glycan moiety, as previously proposed (Hatton et al., 1983; Nair and Robinson, 1999; Hattan et al., 2001), but rather to differences in the amino acid backbone of the EP protein.

Yin et al. (2005) used RT-PCR and cDNA sequencing to determine the primary structure of the EP protein (GenBank accession no. AY364453). Their 213 amino acid sequence was 95.8% identical with that of HIP (SwissProt accession no. P83425), a putative Cd-, Cu- and Zn-binding protein from *M. edulis* blood plasma (Schneeweiss et al., 2002). Independent RP-PCR and cDNA sequencing work in our own lab (Gaudette and Robinson, unpublished data) yielded an incomplete (198 amino acid) sequence for HRG that was 96.5% identical with the corresponding 198 amino acid sequence for EP protein and 95.4% identical with HIP. These results point out the virtual certainty that HIP, EP protein and HRG are identical proteins.

Because HRG (*et ergo,* HIP, EP protein and possibly SPB1) is a dominant protein in the extracellular fluids of *M. edulis* (Robinson et al., 1997; Renwrantz et al., 1998; Nair and Robinson, 1999; Hattan et al., 2001), binds a variety of metals (Renwrantz et al., 1998; Nair and Robinson, 2001a; Hattan et al., 2001), and is involved in the transfer of Cd from the blood to the kidney of the mussel (Nair and Robinson, 2001b), it appears to be a physiologically-important protein. Although first identified in *M. edulis*, we suspected that HRG would also be found in other species of bivalves. We examined this question by analyzing blood plasma from five additional species from two subclasses of Bivalvia, using Cd-Immobilized Metal Affinity Chromatography (IMAC), SDS-PAGE, Western Blotting, and ELISA.

2. Materials and methods

2.1. Organisms

Six marine bivalves were examined (4 pteriomorphs and 2 heterodonts). Blue mussels, *M. edulis* L., (shell length 50–52 mm) were sampled from a low intertidal site in Dorchester Bay, Boston, MA, USA. Horse mussels, *Modiolus modiolus* (L.), (shell length 80–110 mm) were collected subtidally from Saunder's Ledge, off Nahant, MA, USA by divers. *Argopectin irradians* (Lam.) (shell length 69–72 mm) were obtained from Nantucket Harbor, Nantucket, MA, USA. *Mercenaria mercenaria* (L.) (shell length 52–60 mm), *Crassostrea virginica* (Gmelin) (shell length 72–84 mm) and *Mya arenaria* L. (shell length 54–61 mm) were purchased at various times from either James Hook & Co, Boston, MA or Homeport Seafoods, Beverly, MA, USA. After cleaning off epibionts and draining seawater from the mantle cavity, tissue was dissected from the shell and whole blood (including a minor contribution from extrapallial fluid) allowed to drain for 30 min into acid-cleaned centrifuge tubes placed in an ice bath. Whole blood samples were centrifuged in a refrigerated Sorvall RC-5B centrifuge (4 °C) to remove blood cells and tissue debris (10 min at 5900 ×*g*, 15 min at 12,000 ×*g*). Cell-free plasma was frozen in liquid nitrogen and stored at −80 °C until needed. Pooled plasma samples (N=6 to 40 animals) were obtained for IMAC experiments; replicate samples from individual organisms (N=3) were used for ELISA and Western Blotting.

2.2. Immobilized Metal-ion Affinity Chromatography (IMAC)

The Cd-IMAC procedure followed that of Nair and Robinson (1999), except that a much smaller amount of iminodiacetate-Sepharose 6B Fast Flow (IDA; Sigma I4510, 22–30 μmole Zn mL^{-1} metal-binding capacity) was slurry-packed into a tared polypropylene column (40×8 mm; 2–3 g wet wt of packing material). The column was drained, weighed, washed with deionized Milli-Q water (3 column volumes), and charged with 1 column volume of 50 mM $CdCl_2$ (minimum 1 h static equilibration). Excess metal was removed from the column by washing with 2 column volumes of water (20 mL), followed by equilibration with 50 mM HEPES buffer containing 1 M NaCl (pH 7.5; ~3 column volumes). Cell-free blood plasma (2–3 mL) was introduced onto the Cd-conditioned column. The volume of plasma was chosen to ensure that HRG, if present in the blood, would saturate the available binding sites on the IDA. The column was washed with equilibration buffer at a flow rate of approximately 20 mL/h to remove unbound or

weakly-bound proteins. One-milliliter fractions were collected, and their absorbency monitored at 280 nm on a Konitron UVIKON 860 spectrophotometer. After extensively washing the column, bound protein(s) were eluted with 50 mM EDTA. Washings and elutions were carried out until the absorbance of the fractions reached background levels. Fractions from each peak were combined, desalted by dialysis against distilled water using Spectra/Por-6 MWCO 1000 dialysis tubing and lyophilized. Subsamples from each of the combined fractions from each peak, and from the original plasma sample, were taken for protein analysis (Bradford, 1976) and for gel electrophoresis.

The equilibrium metal speciation model MINTEQA2 v4.02 (EPA, 1999) was used to estimate the amount of Cd that bound to each IDA column following the charging and initial wash steps. Cd-IDA and Cd-IDA_2 stability constants (Martell et al., 1998; log K_a=6.57 and 10.96 respectively; 25 °C, 0.0 M ionic strength) were added to the MINTEQA2 thermodynamic database. The amount of protein recovered in the EDTA elution was normalized to the estimated metal content of the column packing (i.e. μg protein/μmole Cd), as well as to the weight of packing material (μg protein/g IDA).

2.3. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples of each bivalve's blood plasma and lyophilized IMAC elutions were analyzed by discontinuous SDS-PAGE (Laemmli, 1970). Lyophilized samples were reconstituted in approximately 50 μL of sample buffer (0.125 M Tris–HCl buffer (pH 6.8), containing 4% (w/v) SDS, 20% (v/v) glycerol, 8.7% (v/v) 2-mercaptoethanol and 0.02% (w/v) bromophenol blue). Plasma samples were mixed 1:1 with sample buffer. Samples were heated in a boiling water bath for 3 min, cooled, and then loaded onto a 3% (w/v) stacking gel/7.5% (w/v) resolving gel (15×15×0.15 cm) and electrophoresed for approximately 3 h (15 °C; 50 mA stacking; 100 mA resolving). Following electrophoresis, gels were either stained with Coomassie brilliant blue R-250 or used for western blotting.

2.4. Production of rabbit anti-HRG antibody

Polyclonal antibodies to purified mussel HRG were raised in two female New Zealand rabbits by Alpha Diagnostic International, San Antonio, TX, USA, using a standard 63-day immunization and bleed protocol (injections at 15 day intervals at multiple (2–4) subcutaneous sites and one intramuscular site). Initial immunization was done by injecting a total volume of 0.5 mL (20–200 μg HRG) in complete Freund's adjuvant. Subsequent injections were given in incomplete adjuvant. Sera were shipped overnight to our laboratory on dry ice and stored at −80 °C upon arrival.

Anti-HRG polyclonal antibodies were purified by ammonium sulfate precipitation and protein A Sepharose chromatography following Crowther (1995). Two milliliters of saturated ammonium sulfate solution was added to 3 mL of rabbit serum containing anti-HRG polyclonal antibody (40% (w/v) ammonium sulfate saturation) and stirred for 30 min followed by centrifugation at 10,000 ×g in a Sorvall RC-5B refrigerated centrifuge for 15 min. The supernatant was discarded and the pellet redissolved in 5 mL of 10 mM phosphate buffer saline (PBS) pH 7.5 and dialyzed exhaustively against three changes of PBS.

Protein A Sepharose (5 mL), packed in a polypropylene column (40×8 mm), was equilibrated with 5 volumes (25 mL) of PBS. Two milliliters of the ammonium sulfate fraction was loaded onto the column, and washed with 5 volumes of PBS to remove unbound proteins. Fractions (1 mL) were collected and absorbances read at 280 nm. Washing was continued at a flow rate 5 mL/min until no protein was detected in the effluent. Elution of bound polyclonal antibody was achieved using 3 volumes (15 mL) of 0.1 M citric acid (pH 3.0). Fractions (1 mL) were collected and neutralized by adding 100 μL of 1 M Tris–HCl (pH 9.0). The purified polyclonal antibody was used immediately or stored at −20 °C in 50% glycerol.

2.5. Western blotting

The specificity of the anti-HRG antibody was evaluated by Western Blotting before use in ELISA (Hylland et al., 1995). Blood plasma was electrophoresed (duplicate gels) on 7.5% (w/v) SDS-PAGE (Laemmli, 1970) or non-denaturing PAGE (Hames and Rickwood, 1986). Afterward, gels were soaked for 20 min in 25 mM Tris–HCl, pH 8.3 containing 192 mM glycine and 15% (v/v) methanol (transfer buffer), and blotted onto polyvinyl diflouride transfer membranes (100 d.c.v. for 2 h; Towbin et al., 1979). After transfer, one of the duplicate blots was stained in 0.1% (w/v) amido black 10B in 10% (v/v) methanol and 2% (v/v) glacial acetic acid for 10 min, destained in 45% (v/v) methanol and 7% (v/v) glacial acetic acid for 10 min, and air-dried. The duplicate blot was placed in a Kapak sealed pouch containing 10 mL Tris blocking buffer (20 mM Tris–HCl, 150 mM NaCl (pH 7.3) 0.1% (v/v) Tween-20; plus 5% (w/v) nonfat dried milk) and incubated on a rocker plate for 1 h at room temperature. The blot was then incubated with 10 mL anti-HRG antibody (a 1:20,000 dilution, diluted in blocking buffer) on a rocker plate for 1 h at room temperature. After three 10-min washes in 10 mL Tris blocking buffer, the blot was incubated in 10 mL horseradish peroxidase conjugated goat anti-rabbit Ig G, diluted in a 1:20,000 dilution in blocking buffer for 1 h at room temperature, washed three times, and briefly rinsed in a solution containing Tris without Tween-20. The blot was then treated with 3 mL 3, 3′, 5, 5′-tetramethyl benzidine ready to use liquid substrate system for membranes (Sigma T-0565) and incubated for 15 min on a rocker plate at room temperature. Positive reactions appeared as dark blue bands against a light purple background.

2.6. ELISA conditions

ELISA was optimized for purified HRG and *M. edulis* blood plasma (Crowther, 1995: 1:20,000 dilution of blood plasma; 1-h incubation (room temperature) with anti-HRG antibody; 30 min incubation (room temperature) with horseradish peroxidase-conjugated goat anti-rabbit IgG). Detection limit (3σ of the blank readings) was 85 pg/mL plasma. Based on

triplicate standard assays, intra-assay Coefficients of Variation (CV) ranged from 2.0 to 6.6%, whereas inter-assay precision ranged from 1.2 to 4.5% (CV) for the various concentrations tested. The practical working range of the ELISA was 1 to 50 ng/mL. Mussel plasma samples spiked with purified HRG yielded recoveries ranging from 93 to 112% (overall recovery 103%±7% CV).

Aliquots of blood plasma (50 μL), obtained from serial dilutions to bring the final protein concentration to 500 ng/mL using 20 mM Tris–HCl (pH 8.5) containing 0.15 M NaCl (coating buffer), were dispensed into wells of a 96-well microtiter plate, and incubated overnight at 4 °C. Solutions were then aspirated and the microtiter plate was washed twice with 200 μL of 20 mM Tris–HCl (pH 7.3) containing 0.15 M NaCl and 0.01% (v/v) Tween-20 (washing buffer). The plate was then blocked by dispensing 100 μL of 20 mM Tris–HCl (pH 7.3) containing 0.15 M NaCl, 0.01% Tween-20 and 5% (w/v) nonfat dried milk (blocking buffer) and incubated overnight at room temperature. After aspirating the blocking buffer, 50 μL aliquots of anti-HRG polyclonal antibody diluted 1:20,000 in blocking buffer was added to the wells and incubated for 1 h at room temperature. Plates were then washed three times and incubated in 50 μL of goat anti-rabbit IgG antibody conjugated with horseradish peroxidase diluted in blocking buffer (1: 20,000 dilution) for 30 min at room temperature. The unbound conjugate was then removed by washing four times, and 75 μL of 3, 3′, 5, 5′-tetramethyl benzidine (ready to use liquid substrate system for ELISA; Sigma, T-0440) was added to each well. After incubation for 15 min in the dark at room temperature, the reaction was terminated by the addition of 1 N HCl. Absorbance at 450 nm was measured using a Spectral shell plate reader (SLT-Lab instruments). Blanks and individual standard curves were run for each separate batch of samples. All experiments were performed in triplicate. OD values obtained were adjusted based on the values of control wells to measure non-specific binding of reagent and a set of interplate controls included to account for any differences from plate to plate.

2.7. Statistical analysis

SPSS 11 software for windows (Statistical Package for the Social Sciences v.11.0, SPSS Inc. Chicago, IL, USA) was used to construct standard curves and for statistical analysis. One-way analysis of variance (ANOVA) followed by Bonferroni *a posteriori* contrast was used to investigate differences among group means. $P \leq 0.05$ was considered significant.

3. Results

3.1. Immobilized Metal-ion Affinity Chomatography (IMAC)

The IMAC elution profile for the pteriomorph *C. virginica* and the heterodont *M. mercenaria* (Fig. 1) exhibited two protein peaks that absorbed strongly at 280 nm. The first broad peak contained unbound plasma protein as well as excess Cd-binding proteins. Lengthy washing of the column with equilibration buffer yielded no additional peaks. The 50 mM EDTA elution, however, yielded a second peak, which was larger for *C. virginica* than for *M. mercenaria* (Fig. 1). Similar elution profiles were obtained for the pteriomorphs *M. edulis* and *M. modiolus*, as well as for the heterodont *M. arenaria* (data not shown). As with *C. virginica*, the pteriomorph *M. modiolus* yielded a larger elution peak than either of the heterodonts. All of the elution profiles were similar to that originally described by Nair and Robinson (1999) for *M. edulis*, even though the amount of IDA used was scaled down significantly.

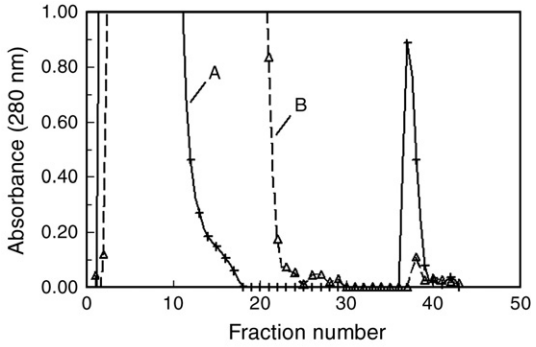

Fig. 1. Immobilized Metal-ion Affinity Chromatography of cadmium-binding proteins from the blood plasma of (A) the pteriomorph *Crassostrea virginica* and (B) the heterodont *Mercenaria mercenaria*. Samples were loaded onto each 40×8 mm iminodiacetate Sepharose column and washed with 50 mM Hepes buffer, pH 7.5, containing 1 M NaCl. Fractions of 1 mL were collected. Elution of bound proteins was achieved by using 50 mM EDTA.

The amount of protein eluted with EDTA from the IMAC column ranged from <1 to 11% of the amount of protein loaded onto the column, and exhibited considerable variability within species (Table 1). 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 within individual species of bivalves or within the entire database (data from all species combined) (Pearson Correlation, $P > 0.05$).

MINTEQA2 modeling estimated that each Cd IMAC column contained 13.8 μmole of Cd per gram of IDA packing material, after being charged and washed with equilibration buffer. EDTA-eluted proteins could therefore be normalized to both the weight of the IDA packing material or to the estimated amount of Cd bound to IDA (Table 1). While some species appeared to have higher amounts of eluted protein, variability was such that there were no significant differences among the five species in either of the normalized protein concentrations (Oneway ANOVA, $P > 0.05$; data log transformed to attain homogeneity of variances at $P \geq 0.01$).

SDS-PAGE confirmed that the EDTA eluted proteins from *M. edulis, C. virginica*, *M. modiolus* and *M. mercenaria* contained one or more protein bands within the range reported for purified mussel HRG (three major polymorphic bands at 35, 37 and 39 kDa, plus a smaller band at 29 kDa; Nair and Robinson, 1999; Table 2). However, no proteins within this range were observed in the plasma from *M. arenaria*. In the other four species, only one or two of the polymorphic bands were typically observed for each species, probably due to the

Table 1
Total protein recovered from Cd-Immobilized Metal Affinity Chromatography (IMAC) experiments on cell-free blood plasma from five species of marine bivalves (selected from two subclasses)

Species	Protein added (mg)	IDA weight (g)	Protein eluted (μg)	Protein eluted/protein added (μg/μg)	Protein eluted/IDA weight (μg/g)	Protein eluted/amount Cd (μg/μmole)
Pteriomorphs						
Mytilus edulis	3.2	2.37	51	0.02	21.5	1.6
	1.9	2.48	219	0.11	88.3	6.4
	2.4	2.47	55	0.02	22.3	1.6
	1.8	2.37	29	0.02	12.2	0.9
	2.4	2.31	49	0.02	21.2	1.5
	1.8	2.13	49	0.03	23.0	1.7
Modiolus modiolus	13.6	2.48	53	<0.01	21.4	1.6
	20.4	2.48	17	<0.01	6.8	0.5
	25.3	2.44	43	<0.01	17.6	1.3
Crassostrea virginica	43.3	2.03	757	0.02	372.9	27.0
	44.1	2.25	472	0.01	209.8	15.2
	46.9	2.33	26	<0.01	11.2	0.8
	34.4	2.42	37	<0.01	15.3	1.1
Heterodonts						
Mya arenaria	28.0	2.67	89	<0.01	33.3	2.4
	26.9	2.37	25	<0.01	10.5	0.8
	27.0	2.18	197	<0.01	90.4	6.6
Mercenaria mercenaria	20.7	2.27	59	<0.01	26.0	1.9
	26.4	2.23	36	<0.01	16.1	1.2
	21.7	2.22	12	<0.01	5.4	0.4

Each row presents the results from a single independent IMAC experiment. Initial column conditions include the amount of plasma added to each column (Protein added; mg), and the weight of the iminodiacetate-Sepharose 6B Fast Flow column packing material (IDA weight; g) for each experiment. Protein eluted (μg) is the amount of protein eluted from the column with 50 mM EDTA. Eluted protein was normalized to the amount of total protein added to the IMAC column (μg eluted protein/μg total protein), to the amount of IDA Sepharose used in each column (μg protein/g IDA Sepharose), and to the amount of Cd bound to each column (μg protein/μmole Cd) estimated using MINTEQA2 equilibrium speciation modeling program (EPA, 1999). Protein content determined by Bradford's (1976) dye-binding assay.

low amount of protein added to the gel (ranging from 37 to 59 μg) and to the presence of additional proteins in the EDTA eluents of most of these species (see below). The 29 kDa band reported by Nair and Robinson (1999) was not observed in the eluents (or in samples of fresh plasma).

EDTA eluents from *M. edulis* exhibited only a single protein band (39 kDa) on 7.5% SDS-PAGE, whereas between 2 and 5 proteins were eluted from the IMAC column in addition to the putative HRG protein for the other four species (Table 2). Both of the heterodonts *M. arenaria* and *M. mercenaria* contained four or five additional proteins, including three that they held in common (60, 57, and 52 kDa). The fillibranch *C. virginica* also displayed the same three common bands (plus bands at 30 and 23 kDa) while *M. modiolus* exhibited two of the common bands (57 and 52 kDa; Table 2). In many cases, these additional bands stained stronger than the 35–39 kDa bands. In *C. virginica*, for example, bands at 30 and 23 kDa stained more strongly than the 39 kDa band. In *M. modiolus* and *M. mercenaria* the 57 and 52 kDa bands stained more strongly than the putative HRG bands. The dominantly stained band in *M. arenaria*, where the

Table 2
Molecular masses of various protein bands observed on 7.5% SDS-PAGE of IMAC EDTA eluents and cell-free blood plasma of six bivalve species

Species	IMAC EDTA eluents			Blood plasma
	Protein loaded (μg)	M_r of putative HRG	M_r of additional proteins	M_r of putative HRG
Pteriomorphs				
Mytilus edulis	51	39*	NO	39, 35*, 34
Modiolus modiolus	53	39	57*, 52*	39, 35*, 34
Crassostrea virginica	37	39	60, 57, 52, 30*, 23*	39, 34*
Argopecten irradians	NAI	NAI	NAI	41*, 39
Heterodonts				
Mya arenaria	40	NO	60, 57, 52, 46*	39, 34*
Mercenaria mercenaria	59	39, 36	60, 57*, 52*, 30, 23	39, 35*

NAI = Not Available for IMAC.
NO = None Observed.
All molecular masses (M_r) are in kDa, and are estimated to fall within a range of ±2 kDa. The dominantly staining protein band(s) in the EDTA eluent and in the blood plasma of each species are indicated with an asterisk.

35–39 kDa protein was not observed, was found at 46 kDa. In most cases, those proteins from the eluent sample that stained dominantly did not stain strongly in samples of cell-free blood plasma (not subjected to IMAC).

3.2. SDS-PAGE of blood plasma

Cell-free blood plasma from all six of the bivalves tested by SDS-PAGE displayed one or more proteins near or within the 35–39 kDa range that characterized mussel HRG (Table 2 and Fig. 2; Nair and Robinson, 1999). *M. edulis* and *M. modiolus* displayed three bands (39, 35 and 34 kDa), with the 35 kDa band staining the strongest. Two bands were observed for *C. virginica* (39 and 34 kDa) and for the two heterodonts (39 and 35 kDa in *M. mercenaria*; 39 and 34 kDa in *M. arenaria*; with the lower molecular mass protein staining predominantly). *A. irradians* also exhibited two protein bands, but at a slightly higher molecular mass (41 and 39 kDa; with the 41 kDa band staining strongest).

3.3. Western blotting

Western blotting, using polyclonal antibodies to purified mussel HRG, confirmed the presence of an HRG reactive protein in all six bivalve species (Fig. 2). As importantly, it demonstrated that the anti-HRG polyclonal antibodies only react with HRG in *M. edulis* and with the 2–3 proteins between 34 and 39 kDa in *M. modiolus, C. virginica*, *A. irradians*, *M. arenaria* and *M. mercenaria*.

3.4. ELISA

Having demonstrated the specificity of the polyclonal antibodies to HRG-like proteins, ELISA detected quantifiable amounts of the HRG in all six of the bivalve species studied, with mean concentrations ranging from 3.09 μg/μL for *A. irradians* to 4.89 μg/μL for *M. edulis* (Fig. 3). Total plasma protein concentrations also varied among the species, with the highest concentrations in the two mussels (7.67 to 8.03 μg/μL) and in the oyster (8.41 μg/μL), and the lowest concentration in the bay scallop *A. irradians* (5.64 μg/μL; Fig. 3). In all species except *A. irradians*, HRG alone constituted the majority of protein in the blood plasma (ranging from 53% in *C. virginica* to 61% in *M. edulis*). In *A. irradians*, the 41% contributed by HRG still makes it the dominant protein present, since there are multiple proteins present in the plasma, none of which are as relatively abundant as HRG. HRG concentrations in the six bivalve species correlated directly with total plasma protein concentrations (Pearson's Correlation Coefficient; $P<0.05$, $N=18$).

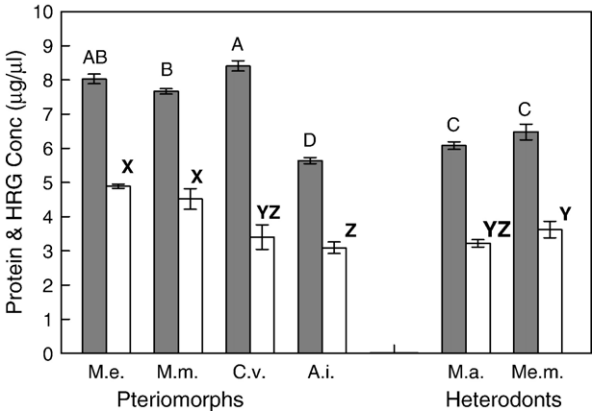

Fig. 3. Mean total plasma protein concentrations (μg/μL; dark bars) and HRG concentrations (μg/μL; light bars) in six bivalve species. Error bars = S.D.; $N=3$. Bars designated with the same letters represent means that are not significantly different ($P \geq 0.05$; Oneway ANOVA followed by Bonferroni *a posteriori* contrast). M.e. = *Mytilus edulis*; M.m.= *Modiolus modiolus*; C.v. = *Crassostrea virginica*; A.i. = *Argopecten irradians*; M.a. = *Mya arenaria*; and Me.m. = *Mercenaria mercenaria*.

4. Discussion

The results presented here support our hypothesis that HRG is normally present in a number of bivalves in addition to the pteriomorph *M. edulis*, where it was originally described. Three lines of evidence were used in making this determination — molecular mass measurements (SDS-PAGE), Cd-binding (IMAC), and recognition by anti-mussel HRG antibodies (Western blots and ELISA). All three criteria were fulfilled for the pteriomorphs *M. edulis, M. modiolus* and *C. virginica*, and for the heterodonts *M. arenaria* and *M. mercenaria*.

Although *A. irradians* were not available for IMAC experiments, the polyclonal antibodies to purified mussel

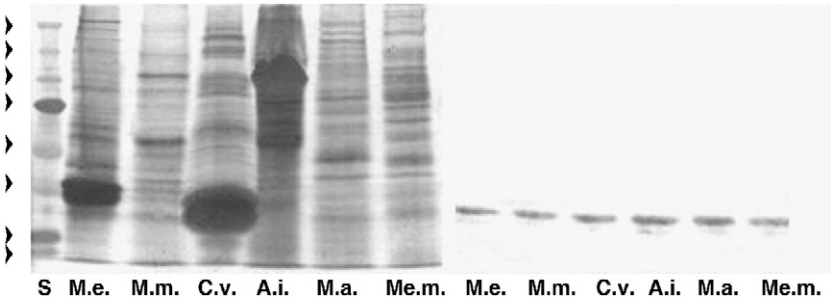

Fig. 2. Identification of HRG from the blood plasma of six bivalve species on PVDF blots following 7.5% SDS-PAGE. Left-hand blot was stained with amido black 10B. Right-hand blot was incubated with anti-mussel HRG antibody followed by horseradish peroxidase-coupled goat anti-rabbit IgG. S = recombinant standard molecular mass markers (from top to bottom M_r 250, 150, 100, 75, 50, 37, 25, and 20 kDa). M.e. = *Mytilus edulis*, 15 μg total plasma protein; M.m.= *Modiolus modiolus*, 25 μg; C.v. = *Crassostrea virginica*, 20 μg; A.i. = *Argopecten irradians*, 20 μg; M.a. = *Mya arenaria*, 30 μg; and Me.m. = *Mercenaria mercenaria*, 30 μg.

HRG, that were specific to HRG in the five other bivalve species, recognized two protein bands in this species. These bands appeared on the protein blots to have a similar molecular mass to the HRG in *M. edulis* and the other four species of bivalves (34–39 kDa; Fig. 2). However, the initial SDS-PAGE separations (reported in Table 2 on different samples than used for the Western Blots) identified possible HRG protein bands of a slightly higher molecular mass (39–41 kDa). This apparent difference points out the run-to-run analytical variability inherent in the analysis of glycoproteins by SDS-PAGE gels. In our experience, it is not uncommon for different investigators as well as the same investigator at different times to obtain as much as a 2 kDa difference in M_r for a protein around 35 kDa in mass. The apparent molecular masses that we report should be viewed with this ±2 kDa variability in mind. A similar variability was observed by Renwrantz et al. (1998; 35±1.8 kDa, $N=6$) and Riffeser and Hock (2002; 35±2 kDa, $N=7$) in their analysis of mussel plasma proteins.

Although we have demonstrated that HRG is present in four pteriomorph and two heterodont bivalve molluscs, it is likely that this protein is even more widespread within the Bivalvia. Robinson and Morse (1994) observed protein bands on SDS-PAGE gels of similar size to mussel HRG in *Mytilus californianus*, *Patinopecten caurinus* and *Crassedoma giganteum* in addition to *M. mercenaria*. More recently, Riffeser and Hock (2002) identified a similar-size protein in *Anodonta cygnea* (as well as in *M. edulis*) using 2-dimensional PAGE. An approximately 36 kDa protein (calculated from the photograph of the published SDS-PAGE gel) was present in the blood of the mussels *Mytilus galloprovincialis* and *Perna australis*, as well as an ~28 kDa protein in the blood of the Pacific oyster *Crassostrea gigas* (Scotti et al., 2001). Unexpectedly, no such HRG-size band was observed in another closely-related Mytilid, *Perna canaliculus* (Scotti et al., 2001). This single negative finding indicated that further studies are needed to determine just how widely distributed HRG is throughout the Bivalvia and the Mollusca.

While a number of other methods could have been used to investigate Cd-binding to HRG in various bivalve species, our choice of IMAC provided at least two intriguing insights that might otherwise have been missed. First of all, only one Cd-binding protein, HRG, was isolated from the blood plasma of *M. edulis* using IMAC, whereas 2–5 additional Cd-binding proteins accompanied HRG in the other 4 species subjected to this procedure. These additional proteins neither reduced nor increased the total amount of protein recovered from the IMAC columns. More importantly though, several of these additionally eluted proteins stained stronger than HRG, implying a higher concentration in the EDTA eluent. Some of these proteins were not major proteins, however, in cell-free blood plasma (not subjected to IMAC). Since ELISA demonstrated that HRG was the dominant protein in the blood of all six bivalves (41 to 61% of total plasma protein), these strongly staining additional proteins must have out-competed HRG for binding sites on the Cd-charged IMAC column. Assuming that this binding did not directly involve the Sepharose packing material or the column walls (unlikely scenarios), these proteins evidently have a much higher affinity for Cd than does HRG ($\log K_a > 5.4–7.6$), and were more efficiently concentrated from the blood samples at the expense of HRG. The recognition of 2–5 as yet uncharacterized Cd-binding proteins opens up several new avenues of research on bivalve metal transport proteins.

The second insight provided by IMAC sheds additional light on the nature of HRG's binding of Cd and potentially other metals. When cell-free blood plasma was subjected to 7.5% SDS-PAGE, either two or three distinct molecular mass HRGs were observed in all six species examined, including a 39 kDa band that was not usually the strongest staining of the HRGs present. In *M. mercenaria*, two HRG bands were observed in both the IMAC EDTA eluent (39 and 36 kDa) and in blood plasma (39 and 35 kDa). However, in *M. edulis, M. modiolus*, and *C. virginica* only the 39 kDa band was present in the EDTA eluents, whereas either the 34 kDa or the 35 kDa band dominated in blood plasma. The IMAC procedure apparently concentrated the 39 kDa isoform in preference to the more prevalent lower molecular mass HRG. Since the microheterogeneity observed in molecular masses of EP protein (thus HRG) has recently been shown to be due to differences in the amino acid backbone of the glycoprotein (Yin et al., 2005) rather than to differences in the size of the glycan moiety (Hatton et al., 1983), the 39 kDa protein may have a modified backbone that allows Cd to bind more tightly than the lower M_r isoforms. Yin et al. (2005) have hypothesized that the differences in the molecular mass of the isoforms arise as a result of post-translational modifications of the EP protein. Our IMAC results may therefore indicate that specific post-translational modifications of the basic HRG backbone regulate the strength of Cd-binding. It is possible that the binding of other metals (e.g. Ca, Cu, Zn) would also vary depending on these post-translational modifications.

In order to determine the function of HRG (*et ergo* EP protein, HIP and possibly SPB1) we need to understand more than just the differences in the strength of metal binding to different isoforms of the protein. More importantly, we need to understand the mechanism for binding and release of different metals. This mechanism may involve metal-specific modifications in the proteins secondary and tertiary structures. Yin et al. (2005) have provided crucial data to support this hypothesis. They reported that both Ca and Cu caused reversible changes in EP protein's secondary structure upon binding. Their Circular Dichroism studies showed that Ca-binding resulted in a reversible loss of β-sheet structure and a concomitant increase in α-helix configuration. Furthermore, they hypothesized that Ca binds to the N-terminal portion of EP protein, while other metals (e.g. Cd) bind to the histidine residues. Since Ca and Cd are thought to bind to different sites on the protein, it is likely that their binding would cause different changes in the protein's secondary and tertiary structures. If different protein configurations are recognized by different receptor molecules on different tissues, this would allow the protein to deliver specific metals to specific target sites within the bivalve. Calcium, for example, may be transported from the blood to the extrapallial fluid and either released at the site of shell mineralization or incorporated into the shell matrix (Hattan et al., 2001; Yin et al., 2005). In addition, the Ca may be transported from

the extrapallial fluid back to the circulatory system during times of anaerobically elevated blood pH (Hattan et al., 2001). Cadmium, on the other hand, may be transported to the kidney and transferred to the kidney cells for subsequent sequestering, detoxification and excretion (Nair and Robinson, 2001b). Upon release of these metals at their target sites, the protein might revert to its original structure and be available for the binding and transport of additional metals. Thus, as already postulated by Yin et al. (2005), the various roles postulated for HRG (metal transport) and EP protein (Ca transport and possibly a shell matrix protein) are not mutually exclusive — this widely distributed protein that is so dominant in blood plasma and extrapallial fluid of pteriomorph and heterodont bivalves, very likely has multiple functions.

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