Purification and characterization of metal-binding proteins from the digestive gland of the Japanese scallop Mizuhopecten yessoensis

Japanese Abstract

ホタテガイの中腸腺由来金属結合タンパク質の分離

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ホタテガイをカドミウム、銅または鉛を含む人工海水に暴露した後、金属の蓄積能および金属結合タンパク質の存在を調べた。いずれの金属も中腸腺に顕著に蓄積され、その蓄積量はカドミウム、銅、鉛の順に高かった。中腸腺から分子量約 28、37 および 42kDa の金属結合タンパク質が精製され、これらのアミノ酸部分配列解析により、*Coccidioides immitis* の calcium-binding protein または *Pleurocapsa* sp. の ion-transporter 類似タンパク質と高い相同性を示すことを明らかにした、これらのタンパク質はホタテガイの金属蓄積または解毒メカニズムに関与していることが示唆された。

Purification and characterization of metal-binding proteins from the digestive gland of the Japanese scallop *Mizuhopecten yessoensis*

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Abstract

Marine bivalves accumulate high concentrations of potentially toxic heavy metals in their tissues. We investigated accumulation patterns of cadmium (Cd), copper (Cu), and lead (Pb) in tissues of the Japanese scallop *Mizuhopecten yessoensis* and clarified that their metal accumulations were associated with certain intracellular metal-binding proteins, after exposure to artificial seawater containing Cd, Cu, or Pb. The scallop was demonstrated to accumulate higher concentrations of Cd than Cu and Pb, and most of the metals were detected in the digestive gland. We purified metal-binding proteins from the digestive gland and performed a preliminary characterization. Three proteins with molecular masses of approximately 28, 37, and 42 kDa were isolated by gel-filtration and anion-exchange column chromatography. Partial amino acid sequences show high sequence similarity to metal-binding proteins and ion-transporters. Metalloprotein profiles in the digestive gland indicated that some proteins were upregulated after metal exposure. We suggest that these proteins are involved in mechanisms of metal accumulation and detoxification in *M. yessoensis.*

Key words: Scallop; heavy metals; bioaccumulation; purification; metal-binding protein

Introduction

Heavy metals are natural components of the Earth's crust, and heavy metals from both natural and anthropogenic sources readily accumulate in marine sediment. This is especially true in coastal zones, which often receive chemical input from many diverse sources of contamination [1]. Heavy metals can enter the aquatic food chain through direct consumption of water or biota and can potentially accumulate at each level of the food chain, which may cause humans to amass a quantity of heavy metals through diet [2]. Therefore, heavy metals are considered a serious environmental threat.

Although marine bivalves have no clear evidence of adaptive immunity [3, 4], they can survive and reproduce in severely contaminated environments for decades and bioaccumulate several metals [5, 6]. Previous research proved that metal-binding proteins called metallothioneins (MTs) play a key role in biochemical detoxification of potentially toxic metals [7, 8]. MTs are well known as low molecular mass (6–7 kDa) cytoplasmic metal-binding proteins that are ubiquitous in eukaryotes [9]. They are also known to play important biological roles such as essential metal homeostasis, detoxification of trace metal ions, and protection against free radicals and intracellular oxidative damage [10, 11]. Recently, considerable research has focused on diversity in inducibility of MTs by metals in bivalves such as oysters *Crassostrea corteziensis* [12] and *Crassostrea gigas* [13], clams *Meretrix meretrix* [14], *Mactra veneriformis* [15], *Scapharca inaequivalvis* [16] and *Cerastoderma*

edule [11], and mussels *Mytilus galloprovincialis* [10] and *Mytilus* sp. [17]. However, until now, the only report on MTs in scallops has been on the bay scallop *Argopecten irradians* [18].

The Japanese scallop, *Mizuhopecten yessoensis* (Jay, 1857), is one of the most economically important marine mollusks living in the cold seas along the coasts of the northern islands of Japan, the northern part of the Korean Peninsula, and the Sakhalin and Kuril islands [19, 20]. Previous studies of *M. yessoensis* focused on metal accumulation and metal bioaccumulation patterns suggested that scallops have evolved a natural capacity to accumulate, detoxify, and store metals in their tissues [21, 22]. Hence, it is necessary to study the ability to bioaccumulate different heavy metals and corresponding metal-binding proteins including MTs.

In the present work, bioaccumulation and tissue distribution of heavy metals in the scallop *M. yessoensis* were examined by using laboratory experiments under controlled conditions. Accumulation of metals was monitored using living shells exposed to seawater containing cadmium (Cd), copper (Cu), and lead (Pb). Furthermore, to clarify the function of the metal-rich proteins in *M. yessoensis* after acute metal exposure, proteins in the digestive gland were purified by gel-filtration and ion-exchange chromatography, and then separated by electrophoresis. Finally, the isolated proteins were characterized by partial amino acid sequence analysis.

Materials and methods

Scallops and metal exposure

Adult Japanese scallops *M. yessoensis* were collected from Tokyo central wholesale fish market, Japan. Scallops (shell length: 11.6 ± 0.3 cm, weight: 193.7 ± 31.0 g) were maintained in aquariums for 7 days prior to experiments. They were fed a commercial diet of *Ultra Clam* (Fauna Marin, Holzgerlingen, Germany), which is a special food for filter feeders without heavy metals and was added every day at a concentration of 0.1 g per 100 l seawater during both acclimatization and exposure periods. Glass tanks with dimensions 90 × 45 × 45 cm were filled with 40 l of synthetic seawater (salinity = 3.3%) and aerated by a diffuser system. Temperature was set at 18 ± 1^{o}C and the photoperiod was fixed at 12 h using artificial light sources. After the acclimation period, 60 scallops were used for metal exposure experiment, and randomly divided into 6 groups, each group with 10 individuals. Cd was added as $CdCl_2 \cdot 2.5H_2O$ at the concentrations of 200 µg/l and 400 µg/l. Cu and Pb were added as $CuCl_2 \cdot 2H_2O$ and $Pb(NO_3)_2$ at the concentrations of 100 µg/l and 200 µg/l, respectively. The water in each tank was changed every two days to ensure no accumulation of toxic materials from the scallops, which could change water quality. As a control, 10 scallops without metals were maintained under similar conditions.

After each sampling time (7 and 10 days), 5 scallops were randomly selected from both the control and metal-treated groups. Tissues of the digestive gland, gill, mantle, gonad, and adductor muscle were collected separately from individual scallop. Tissues for investigation of metal accumulation were completely dried at 80^{o}C for more than 24 h until a constant

weight was reached, and then the dried tissues were stored in a desiccator at room temperature until they were processed. The digestive glands used for protein extraction were stored at –80°C for further use.

Quantification of metals in the tissues of scallops

Approximately 50 mg aliquots of dried tissue was digested with 14 mol/l nitric acid (HNO_3) for 24 h at room temperature and then heated at 80°C for 6 h until totally digested [23]. Milli-Q water was added to the digested samples to dilute the HNO_3 to 2.0% (v/v). Finally, the samples were filtrated with 0.22 μm membranes before atomic absorption spectrophotometry (AAS) measurement. In control and metal-exposed scallops, Cd, Cu, and Pb levels were determined using a Hitachi Z-2000 AAS (Hitachi, Japan). All metal concentrations are given on a dry weight basis (μg/g dry wt), the values are the mean ± SD of five individual experiments performed in triplicate.

Extraction and separation of metalloproteins

Approximately 30 g of the digestive glands from scallops exposed to metals and non-exposed scallops were pooled separately and homogenized in 3 volumes of ice-cold 10 mM Tris-HCl buffer (pH 8.6), which contained 1 mM dithiothreitol (DTT) and 0.1 mM phenylemethylsulfonyl fluoride (PMSF) as an antioxidant and antiproteolytic mixture. The homogenate was centrifuged at 30,000 × *g* for 40 min (4°C). The supernatant (subcellular fraction) containing metalloproteins was partially purified by acetone fractionation (50–80%)

as previously described [24, 25]. Briefly, cold acetone (–20ºC) was added to the supernatant to a final concentration of 50%. The sample was maintained at 4ºC for 30 min with magnetic stirring and then centrifuged at 14,500 × *g* for 10 min (4ºC). The pellet was discarded and the acetone concentration of the supernatant was raised to 80%. The preparation was maintained at 4°C for 40 min with magnetic stirring and then centrifuged again at 14,500 × *g* for 10 min (4ºC). The 80% acetone-precipitated pellet was resuspended in 10 mM Tris-HCl buffer (pH 8.6), which contained 1 mM DTT and 0.1 mM PMSF. Protein concentrations of all samples were measured in triplicate by the Bradford method (Quick Protein Assay, BioRad, Hercules, CA, USA). Metal concentrations in proteins were determined in triplicate by AAS as previously described. Fifty mg of dissolved 50-80% acetone pellet from scallops in the non-exposed group and those exposed to 200 µg/l of $CdCl_2$, $CuCl_2$, or $Pb(NO_3)_2$ was loaded onto a Sephadex G-50 gel-filtration column (2.6 × 100 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.6), that contained 1 mM DTT and 0.1 mM PMSF. The samples were eluted with the same buffer at a flow rate of 0.5 ml/min after sample application. To detect proteins and thiolate-metal linkage in protein, absorbance of the eluted fractions at 280 and 254 nm was monitored continuously with an ultraviolet spectrometry (Shimadzu UV-1800, Shimadzu, Japan) [26]. Cd, Cu or Pb levels were also continuously monitored with AAS. The column was calibrated for molecular weight estimation with bovine serum albumin (66 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), horse cytochrome C (12.4 kDa), and aprotinin from bovine lung (6.5 kDa) (Sigma-Aldrich, St. Louis, MO, USA). Cd-containing fractions were pooled and applied directly onto a Mono Q™ 5/50 GL column (GE Healthcare, Pittsburgh,

PA, USA). The column was equilibrated with buffer A (10 mM Tris-HCl, pH8.6). Following a wash with buffer A, proteins were eluted with a linear gradient of buffer B (250 mM Tris-HCl, pH 8.6) in buffer A at a flow rate of 0.5 ml/min. UV intensity of proteins on 254 nm was monitored by a MD-2010 UV detector (JASCO, Tokyo, Japan). Cd contents of the eluted peaks were determined by AAS as described above. Purified Cd-binding protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and 2-Dimensional (2-D) electrophoresis

Proteins extracted from the digestive glands of scallops were subjected to SDS-PAGE (12.5% acrylamide). For further analysis, 2-D electrophoresis was also performed. Fifty μg aliquot of each protein sample was diluted with 125 μl of rehydration buffer (7.0 M urea, 2.0 M thiourea, 4.0% CHAPS (w/v), 20 mM DTT, 2 mM tributylphosphine and 0.4% (w/v) pharmalyte 3–10), and applied to a 7 cm linear IPG strip (pH 3–10, GE Healthcare). Isoelectric focusing (IEF) was conducted using an Ettan IPGphor Ⅱ system (GE Healthcare) at 300 V for 45 min, 300 to 1000 V for 30 min, 1000 to 5000 V for 1.2 h, and 500 V for 25 min, successively. The focused IPG strips were reduced for 25 min with 1.0% DTT (w/v) in equilibration buffer (50 mM Tris-HCl, 6.0 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.001% (w/v) bromophenol blue, pH 8.8), followed with alkylation for 25 min with 2.5% (w/v) iodoacetamide in the equilibration buffer. After equilibration, the strips were subjected to SDS-PAGE (5–20% acrylamide) at 20 mA per gel. Finally, the proteins were visualized by staining with a Rapid CBB Staining kit (Kanto Chemicals, Tokyo, Japan).

Measurement of ultraviolet absorption spectrum

The Cd-containing fraction obtained from Sephadex G-50 was assessed by ultraviolet absorption spectroscopy from 200 to 400 nm (Shimadzu UV-1800, Shimadzu, Japan). In addition, the Cd-containing fraction was acid-hydrolyzed with 0.1 M HCl and 0.5 M EDTA at room temperature for 20 min, and then its UV spectrum was also examined as described above.

Amino acid sequencing

Following SDS-PAGE, purified protein was transferred to a PVDF membrane, and then stained with CBB. The target bands were cut out and analyzed in a protein sequencer (ABI Procise 491HT, Applied Biosystems, Nippi Research Institute of Biomatrix, Japan).

Data analysis

Values are expressed as mean ± SD. SPSS software (version 20, IBM, Armonl, NY, USA) was used for statistical analysis. Data from control scallops and metal-exposed scallops were compared using one-way analysis of variance (ANOVA) and statistically different treatments were identified using Duncan's test. All differences were considered significant at $p < 0.05$. The letters a, b, c, and d indicate significant differences between groups.

Results

Concentrations of Cd, Cu, and Pb in different tissues of scallops

Concentrations of metal in tissues of control and exposed scallops *M. yessoensis* were measured by AAS (Table 1). In tissues from control scallops, the highest Cd concentration (62.28 ± 42.73 μg/g dry wt; $p < 0.05$) was observed in the digestive gland. This was 14 times higher than that observed in gills (4.23 ± 1.75 μg/g dry wt), which had the second highest concentration. Similarly, the digestive gland displayed the highest Cu (11.69 ± 8.51 μg/g dry wt; $p < 0.05$) and Pb (0.88 ± 0.31 μg/g dry wt; $p < 0.05$) concentrations among tissues, Cu and Pb levels in the digestive gland were 2 times higher than that in gonads, which had the second highest concentrations. Following exposure to metals, metal concentrations in different tissues increased globally. Moreover, the digestive gland displayed highest concentrations for all metals, with the exception of scallops exposed to 100 μg/l of Pb for 7 d. Although a significant increase in Cd concentration in the digestive gland of scallops was observed with 400 μg/l of $CdCl_2$ exposure ($p < 0.05$), there was no significant difference with exposure time ($p > 0.05$). In contrast, Cu and Pb concentrations in the digestive glands of the exposed scallops presented significant differences ($p < 0.05$) and both showed significant differences with exposure time ($p < 0.05$), with the exception of scallops exposed to 100 μg/l of $CuCl_2$ and $Pb(NO_3)_2$ for 7 days. Given that the digestive gland is considered the major tissue that accumulates trace elements in Japanese scallops, the digestive glands of scallops were dissected and homogenized to investigate protein profiles.

2-D electrophoretic analyses

Figure 1 presents electrophoretic analysis of extracts obtained from the digestive glands. SDS-PAGE analysis revealed that some proteins with molecular weights of approximately 100, 50, 42, 37, and 28 kDa were differentially expressed during exposure to heavy metals. The most marked difference among different exposure groups was a band at approximately 42 kDa, which was upregulated when exposed to all three metals. On the other hand, some bands (approximately 100 kDa) were significantly appeared when Cu and Pb ions were exist (Fig. 1a). Further analysis by 2-D electrophoresis also showed a distinctive difference between control scallops and scallops exposed to 200 µg/l of $CdCl_2$ for 7 days, with a protein spot of approximately 42 kDa that had an acidic pI (red arrow in Fig. 1c).

Protein purification

Results of AAS analyses of the digestive gland and its extracts indicated that more than 65% of the total Cd accumulated in the digestive gland was detected in the subcellular fractions, irrespective of metal exposure conditions (Table 2). To purify metal-binding proteins, extracts from the digestive glands of control and metal-exposed scallops were subjected to gel-filtration. Figure 2 shows the Sephadex G-50 column elution profiles of resuspended pellets obtained from the 50–80% acetone precipitations. The profiles at 280 nm indicated two major peaks and a minor peak, the first with relatively high molecular weight (> 25 kDa), corresponding to void volume of the column, the second with molecular weight less than 10 kDa, and the minor peak with low molecular weight (< 1.5 kDa) at around fraction number

100 in Fig. 2c and d, corresponding to bed volume of the column. However, metals Cd, Cu, and Pb were detected at only the first peak, which indicated that these metals were bound to high molecular weight substances.

The Cd-containing fractions were subjected to further purification on Mono Q^{TM} 5/50 GL ion exchange HPLC. Eluted fractions were monitored by absorbance at 254 nm. As shown in Fig. 3a, at least four peaks were eluted at the retention times of 8, 28, 65, and 93 min, and then the unabsorbed fraction, fraction 1, and fraction 2 were pooled separately. The concentrations of Cd in three fractions were 42, 28, and 55 μg/mg protein of Cd, and the Cd amounts of these fractions were 5.7, 7.3, and 33.1 μg, respectively. Therefore, it is indicated that fraction 2 mainly contained Cd-binding proteins. Results of SDS-PAGE analysis revealed that fraction 2 consisted of two clear bands with molecular weights of approximately 42 and 37 kDa, in unabsorbed fraction and fraction 1, there were one clear band with a molecular weight of approximately 37 kDa and some faint bands with molecular weights of approximately 28 and 22 kDa (Fig. 3b).

Characterization of Cd-binding proteins

Two components with relative high molecular weights of approximately 42 and 37 kDa were detected in the main Cd-containing fraction 2, which designated to metal-binding protein (MBP)-42 and MBP-37, respectively. To confirm whether these proteins are related to metal-binding, amino acid sequence analysis was carried out. N-terminal amino acid sequences of MBP-42 and MBP-37 were determined as follows: SAPASNAKLR and

VIIRIFLLRS, respectively. Additionally, MBP-28 from the unabsorbed fraction was determined to be LDEAEFKYQ. A database similarity search using these partial amino acid sequences revealed high identities with some proteins of metallophosphoesterase (WP_011140060.1), metal-binding proteins (EJB10385.1) or ion-transporter (WP_012298547.1) (Table 3).

All of metal-containing fractions eluted from Sephadex G-50 exhibited relatively high UV absorption at 254 nm (Fig. 2), which indicated that metals were linked to the metal-binding protein fractions with high thiol content. The OD254/280 ratio of Cd-exposed scallops was 1.61, which was higher than that of Cu (1.40) and Pb (1.26) (Fig. 2b, c, and d). An UV absorbance spectrum of pooled Cd-containing fractions showed a characteristic pattern in the 200–400 nm range, with a broad peak at 256 nm that is similar to the typical pattern of Cd thiolate cluster [24]. The procedure of treating Cd-rich fractions with HCl at pH 1 removed metal ions from metal-binding proteins, which then led to a red shift of the absorption maximum of its retinal from 256 nm to 267 nm (Fig. 4).

Discussion

The present work confirmed the high metal bioaccumulation potential of the Japanese scallop *M. yessoensis*. The digestive gland is the organ to accumulate metals at the highest levels in the scallop not only in natural but also metal-exposed conditions. In this study, to assess the contamination status and bioaccumulation ability of the Japanese scallop *M. yessoensis*,

concentrations of Cd, Cu, and Pb were analyzed in relative to metal exposure (Table 1). Interestingly, in the normal state without metal exposure, the digestive gland accumulated most of the elements. In contrast, other tissues seemed to play a minor role in the storage of Cd, Cu, and Pb, although they may play a major role in uptake and transfer of trace elements [27]. Previous studies have pointed out the ability of various scallop species to accumulate high trace elements in their tissues, even in remote areas such as Antarctica or Arctic Oceans where are not subjected to direct anthropogenic inputs [25, 28, 29]. Furthermore, it is reported that Cd in the digestive glands of 1–8 year-old *M. yessoensis* collected from the Sea of Japan increased from 39 to 400 µg/g dry wt, but in the muscle, mantle and gill did not exceed 6 µg/g dry wt in the oldest scallop [30]. This suggests either a higher Cd bioaccumulation capacity of this scallop species and/or lower bioavailability of Cu and Pb for *M. yessoensis* in fields of cultured scallop. Therefore, we compared bioaccumulation ability of the scallops by exposure to Cd, Cu, and Pb at the concentration of 200 µg/l. Following exposure, metal concentrations in all scallop tissues increased globally and most of metals were detected in the digestive gland where the concentration of Cd was significantly higher than that of Cu and Pb ($p < 0.05$, Table 1). This indicates that *M. yessoensis* can accumulate different metals such as Cd, Cu, and Pb, but that the bioaccumulation ability differs such that: Cd > Cu > Pb.

The concentration of Cd in the digestive gland of *M. yessoensis* is age-specific [21]. Cd in the cytoplasmic fraction of the digestive gland is also age-specific; 71.7% of this metal was detected in subcellular fractions of the digestive glands from l-year-old scallops, compared with 98.8% in 8-year-olds [30]. In our study, more than 65% of metals were found in the

cytoplasmic protein fraction of the digestive gland, and the metals were likely bound with high molecular weight substances like proteins. Previous studies reported that Cd was bound with different kinds of proteins in the hepatopancreas of *M. yessoensis*, including metallothionein (MT)-like proteins and high molecular weight proteins (HMWP) [8, 30, 31]. Nakayama and co-workers (1995) noted that most of the accumulated Cd existed in the soluble fractions of hepatopancreas, with molecular weights of approximately 40 and 30 kDa. More recent work reported the existance of two Cd-binding proteins with molecular weights of 72 and 43 kDa in *M. yessoensis*, which are thermally stable [8]. Although the 40 and 30 kDa materials were not purified and the characteristics of 43 kDa Cd-binding protein were not fully elucidated, it seems that some of them probably are identical to the purified proteins found in this study. In the digestive gland of the Antarctic scallop *Adamussium colbecki*, Cd was associated with MT only (about 10 kDa) [25]. However, our results showed that in the digestive glands of Japanese scallop *M. yessoensis*, not only Cd but also Cu and Pb were bound to only HMWPs (> 25 kDa), but not in MT fraction.

In our study, three proteinaceous components with molecular weights of approximately 42, 37, and 28 kDa were isolated as possible metal-binding proteins. Database similarity search using their partial amino acid sequences revealed identities with several proteins related metal-binding and ion-transport proteins, such as metallophosphoesterase which contains two ions in a typical dinuclear center [32] and heavy metal ion-transporters play roles in metal uptake, regulation of metals, and export of metals [33]. Although information on these proteins does not appear to be available for bivalves, it is possible that these proteins isolated

newly are involved in the metal accumulation and detoxification in *M. yessoensis*, since MTs were not found in the digestive gland of scallop. Further investigations on the primary structures and characteristics of these proteins are in progress to elucidate the underlying mechanisms.

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Figure captions

Fig. 1 Electrophoretic analyses of metalloproteins extracted from the digestive glands of scallops. **a**: Ten µg of protein analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). **b** and **c**: Isoelectric focusing (IEF) analysis of 50 µg of protein from control scallops (**b**) and 50 µg of protein from scallops exposed to 200 µg/l of Cd for 7 days (**c**). Gels were stained using CBB staining kit. Red arrows indicate a protein with a molecular weight of approximately 42 kDa.

Fig. 2 Chromatographic profiles (Sephadex G-50) of resuspended acetone precipitate from the digestive gland of scallops in which gel-filtration chromatography was monitored at 280 and 254 nm. **a**: control, **b**: 200 µg/l Cd for 7 days, **c**: 200 µg/l Cu for 7 days, **d**: 200 µg/l Pb for 7 days.

Fig. 3 Anion-exchange chromatogram on a Mono Q column detected by UV detector at 254 nm (**a**) and 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified proteins (**b**). The samples were obtained using a Sephadex G-50 column. M: marker, Un: Unabsorbed fraction from anion-exchange chromatography, 1 and 2: fractions 1 and 2 collected from anion-exchange chromatography. Red arrows indicate target proteins of MBP-42, MBP-37, and MBP-28.

Fig. 4 Absorbance spectrum of Cd-binding proteins purified using a Sephadex G-50 column. Black line: Cd-binding proteins, red line: Cd-binding proteins treated with HCl.

Figure 1

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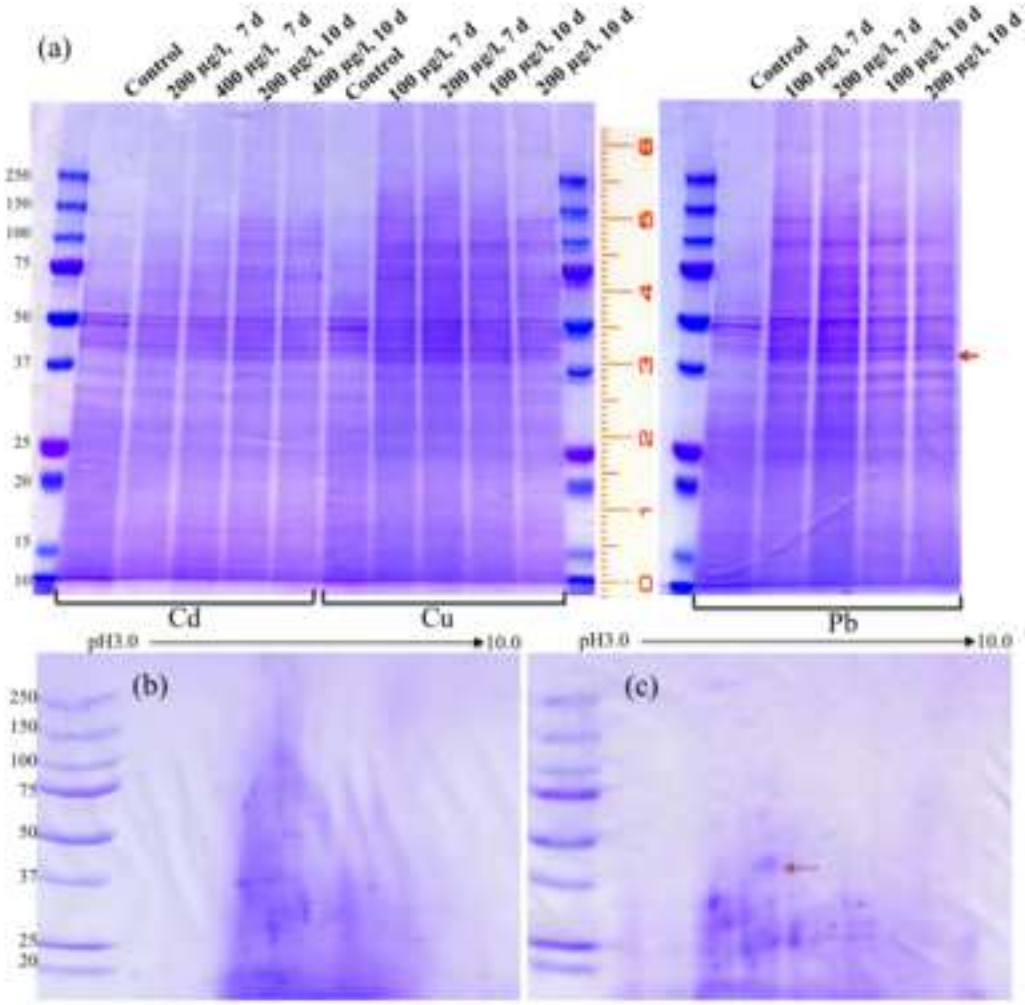

Figure 2

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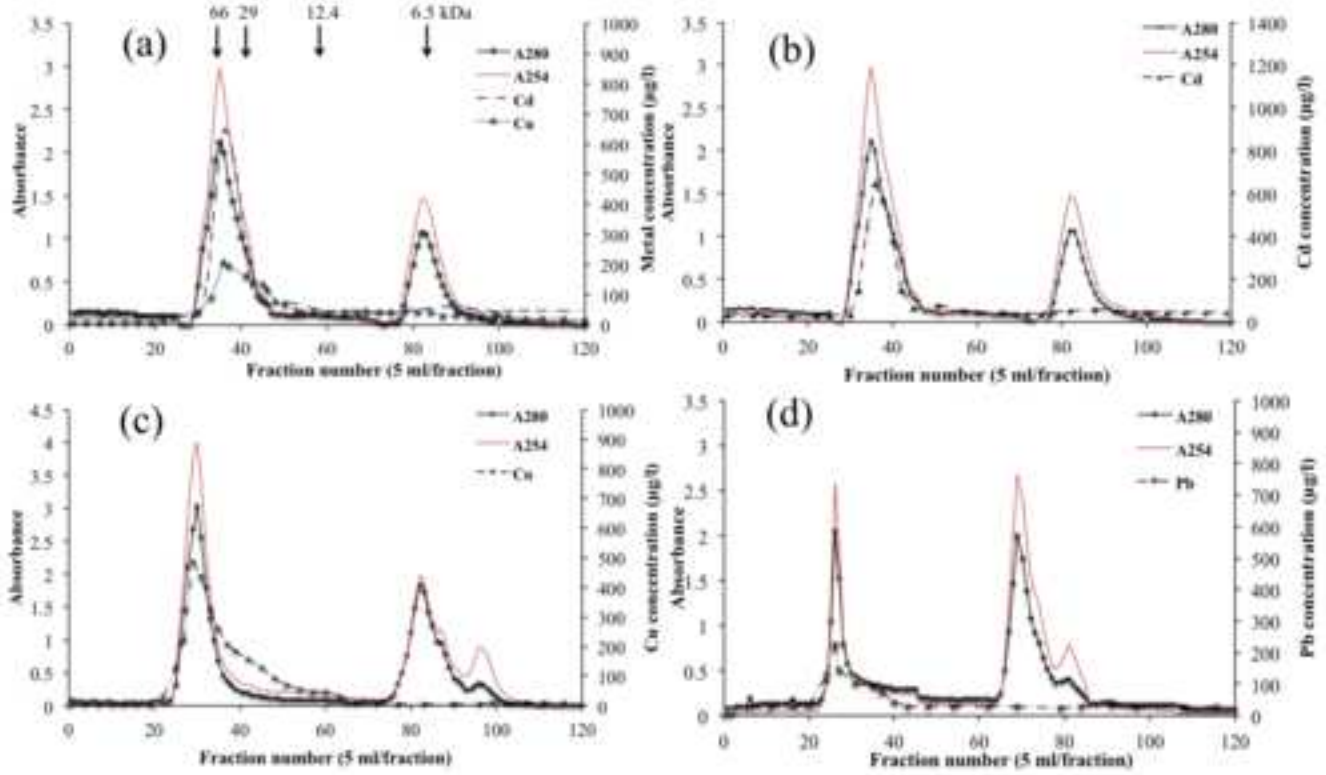

Figure 3

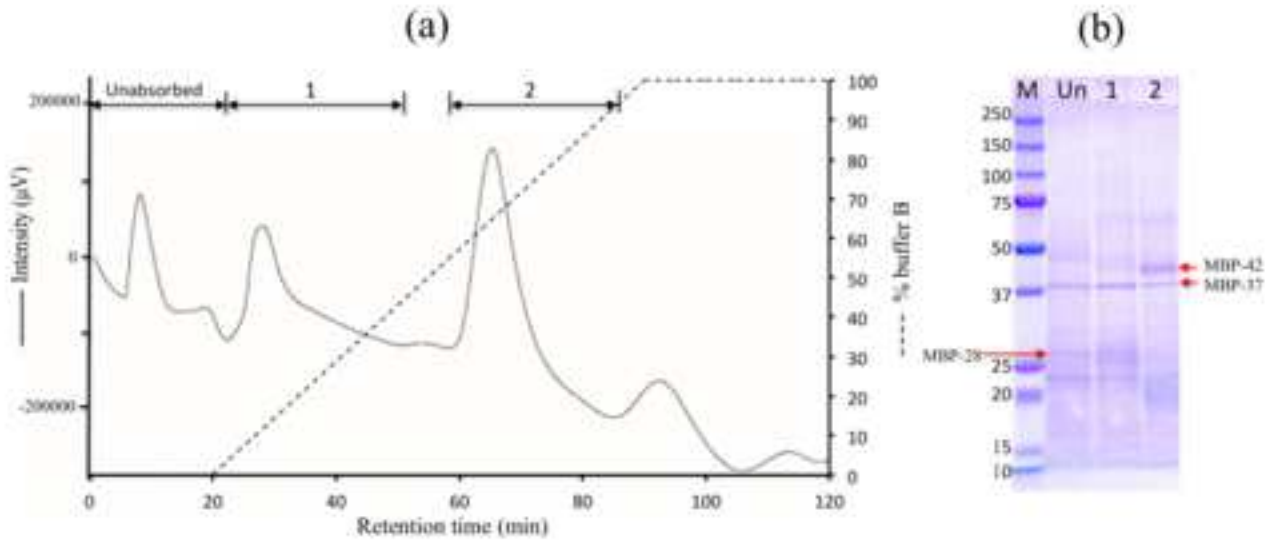

Figure 4

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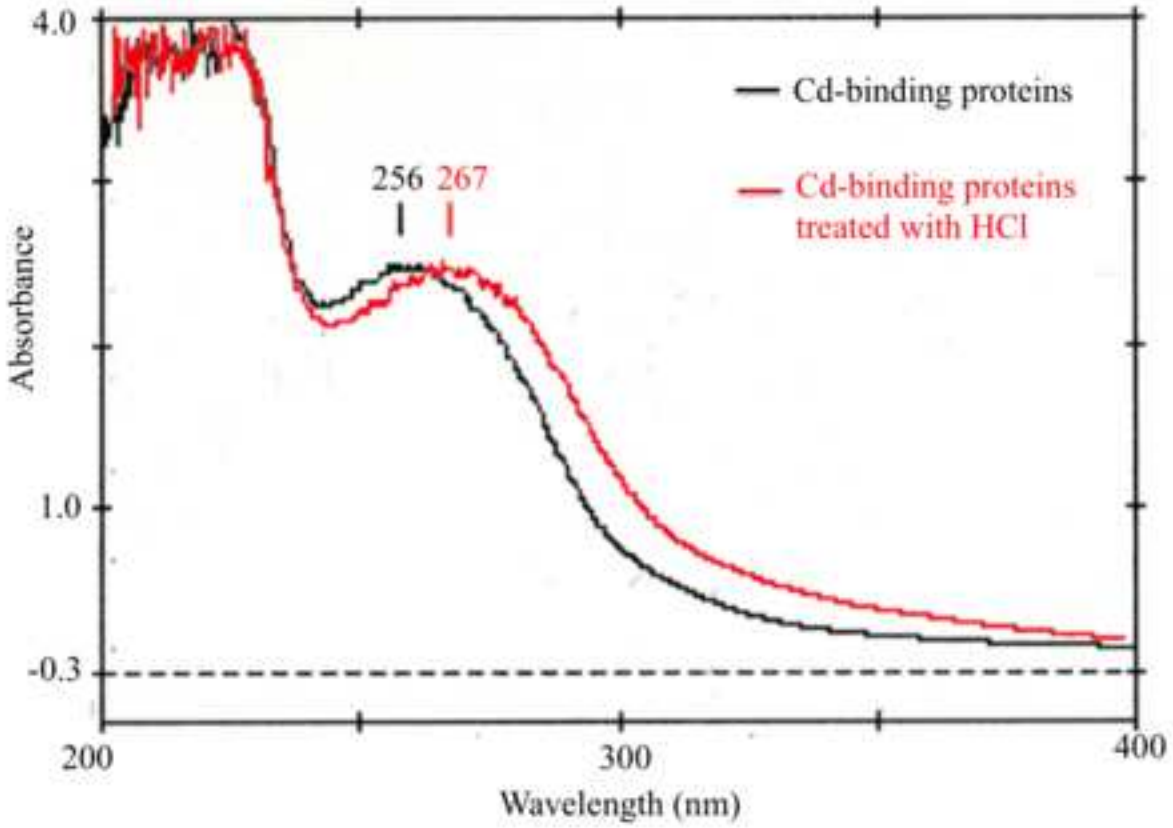

Table 1 Metal concentrations (μg/g dry weight) in different tissues of scallops.

Exposure condition		Tissues				
		Digestive gland	Mantle	Gill	Gonad	Adductor muscle
Cd	0	62.28 ± 42.73^{a}	2.13 ± 0.89^{a}	4.23 ± 1.75^{a}	1.16 ± 0.35^{a}	0.57 ± 0.19^{a}
	200 μg/l, 7 d	122.49 ± 13.58^{ab}	15.70 ± 7.67^{b}	70.34 ± 37.15^{abc}	6.43 ± 2.89^{ab}	2.16 ± 0.93^{ab}
	400 μg/l, 7 d	180.99 ± 42.64^{b}	28.66 ± 11.60^{bc}	50.26 ± 14.82^{ab}	14.26 ± 7.97^{b}	2.34 ± 1.43^{b}
	200 μg/l, 10 d	150.79 ± 18.37^{ab}	18.48 ± 3.40^{bc}	89.08 ± 15.52^{bc}	11.68 ± 4.16^{b}	2.77 ± 0.47^{ab}
	400 μg/l, 10 d	217.36 ± 107.48^{b}	31.45 ± 5.36^{c}	110.66 ± 37.80^{c}	28.04 ± 5.62^{c}	5.82 ± 0.38^{c}
Cu	0	11.69 ± 8.51^{a}	0.88 ± 0.07^{a}	3.10 ± 2.70^{a}	4.17 ± 1.38^{a}	0.53 ± 0.08^{a}
	100 μg/l, 7 d	31.33 ± 20.90^{ab}	1.24 ± 0.23^{a}	16.65 ± 12.19^{ab}	5.41 ± 1.03^{ab}	0.81 ± 0.21^{ab}
	200 μg/l, 7 d	64.24 ± 28.05^{b}	3.98 ± 0.82^{b}	44.32 ± 27.01^{bc}	8.65 ± 0.57^{cd}	1.61 ± 0.14^{c}
	100 μg/l, 10 d	53.11 ± 8.16^{b}	2.86 ± 0.92^{b}	40.82 ± 18.00^{bc}	7.22 ± 0.75^{bc}	1.07 ± 0.13^{b}
	200 μg/l, 10 d	125.46 ± 27.1^{c}	6.18 ± 0.59^{c}	61.89 ± 28.12^{c}	10.27 ± 1.20^{d}	2.88 ± 0.52^{d}
Pb	0	0.88 ± 0.31^{a}	0.25 ± 0.06^{a}	0.17 ± 0.05^{a}	0.40 ± 0.25^{a}	0.08 ± 0.04^{a}
	100 μg/l, 7 d	5.24 ± 2.80^{ab}	0.59 ± 0.14^{a}	7.04 ± 3.26^{ab}	2.19 ± 1.08^{ab}	0.20 ± 0.04^{a}
	200 μg/l, 7 d	16.13 ± 5.08^{b}	1.56 ± 0.67^{a}	10.34 ± 3.10^{ab}	2.89 ± 0.71^{b}	0.72 ± 0.06^{b}
	100 μg/l, 10 d	15.85 ± 4.28^{b}	1.66 ± 0.87^{a}	12.14 ± 4.70^{bc}	2.29 ± 0.52^{ab}	0.45 ± 0.10^{c}
	200 μg/l, 10 d	40.91 ± 12.39^{c}	4.01 ± 2.34^{b}	21.39 ± 10.21^{c}	5.69 ± 1.84^{b}	1.03 ± 0.19^{d}

Cadmium (Cd), Copper (Cu) and Lead (Pb) concentrations are the mean ± SD of five individual experiments performed in triplicate. Significant differences ($p < 0.05$) in metal concentration of each tissue among different exposure conditions are indicated with letters (a, b, c, or d).

Table 2 Cadmium (Cd) distributions in subcellular fractions from the digestive glands of the scallops.

	Digestive gland		Subcellular fraction	
	Wet weight (g)	Total Cd (μg)	Total protein (mg)	Total Cd (μg)
Control	26.3	356.7 ± 31.1	468.4 ± 43.5	232.4 ± 14.3 (65.2%)
200 μg/l, 7 d	28.6	767.1 ± 46.3	646.7 ± 36.9	503.4 ± 43.7 (65.6%)
400 μg/l, 7 d	21.8	868.3 ± 59.7	480.6 ± 31.4	587.4 ± 62.3 (67.6%)
200 μg/l, 10 d	24.3	806.4 ± 66.4	734.3 ± 62.5	565.6 ± 55.8 (70.1%)
400 μg/l, 10 d	24.7	1181.7 ± 91.8	784.4 ± 58.7	821.5 ± 74.2 (69.5%)

Cd and protein contents are mean ± SD of pooled sample performed in triplicate, numbers in （）indicate mean of Cd content as percent total Cd in the digestive gland.

Table 3 Database similarity search using N-terminal protein sequences.

Sequence name	Amino acid sequence	Protein name	NCBI Accession no.	Matched amino acid count
MBP-42	SAPASNAKLR	Metallophosphoesterase	WP_011140060.1	8/10
MBP-37	VIIRIFLLRS	Ion-transporter	WP_012298547.1	7/10
MBP-28	LDEAEFKYQ	Calcium-binding protein	EJB10385.1	7/10