Cadmium-Binding Proteins of Three Marine Molluscs and Characterization of Two Cadmium-Binding Glycoproteins from the Hepatopancreas of a Whelk, *Buccinum tenuissimum*

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The cadmium-binding proteins were shown to exist in the hepatopancreas of three molluscs, a whelk, *Buccinum tenuissimum*, a turbo, *Batilllus cornutus*, and a squid, *Todarodes pacificus*. Cadmium was efficiently accumulated in nature to a mean concentration of 119, 33, and 50 μg/g wet tissue in the hepatopancreas of three species of molluscs, and 30%, 11%, and 43% of the element in each tissue of whelk, turbo, and squid was extracted to the soluble fraction, respectively. Separation of the soluble fraction by Sephadex G-75 in the presence of 2-mercaptoethanol revealed that cadmium was mainly bound to the protein fraction FII of molecular weight 10,000.

Two cytoplasmic cadmium-binding glycoproteins from the hepatopancreas of *Buccinum tenuissimum* were purified to homogeneity by Sephadex G-75 gel filtration and double DEAE–Sephadex A-25 chromatographies in the presence of 2-mercaptoethanol. These two cadmium-binding glycoproteins, termed FIIα and FIIβ, had molecular weights of 8000 and 13,000 and consisted of 52 and 94 amino acid residues, respectively. Three and two cysteine residues in FIIα and FIIβ, respectively, were found and two more half-cystine were also detected in FIIβ. The sugar contents of FIIα and FIIβ were about 20.5% and 8.7% by weight, respectively, consisting of galactose, mannose, fucose, and amino sugar. Both showed strong metal-binding ability, especially for cadmium, copper, and mercury.

Introduction

An accumulation of cadmium in various living organisms has attracted the attention of many investigators due to its chronic effect on health. Since the element is widely distributed in nature, many animal and plant tissues become major possible sources for accumulation of the metal in human being. Degree of absorption and storage of cadmium are dependent on its chemical form in various food resources (1). Although bioaccumulation in the hepatopancreas of edible marine molluscs was reported (2,3), there have been no data to answer the question whether this element might be a normal constituent of living matter or only a spurious contaminant.

Recently, metallothionein isoproteins have been isolated in some invertebrate; crustaceans and zooplanktor (4). We have found hepatopancreas of a whelk, *Buccinum tenuissimum*, to be rich source of heavy metal-binding proteins (5,6). The study described here provides molecular properties of two cadmium-binding glycoproteins from whelk hepatopancreas, which are significantly different from mammalian metallothionein, together with the evidence for the existence of cadmium-binding proteins in the hepatopancreas of two more molluscs.

Materials and Methods

Materials

Whelk, *Buccinum tenuissimum*, turbo, *Batilllus cornutus* and squid, *Todarodes pacificus*, were collected from off the west coast of Japan Sea, Japan. Hepatopan-
creas of these molluscs was removed from extraneous tissues immediately and soaked in saline.

Sephadex G-25, G-75 and DEAE–Sephadex A-25 were purchased from Pharmacia Fine Chemicals, and ampholine for the pH range between 3.5 and 10.0 from LKB Produktor, AB. Tris was obtained from Sigma Chemical Co. Analytical-grade cadmium chloride and other metal salts were obtained from Wako Pure Chemicals. All dialyses were performed with Spectrapor 1 or Spectrapor 3 membrane tube (Spectrum Medical Industries, Inc.) after being immersed in 0.01 M EDTA solution.

Methods

Whelk and squid hepatopancreas were homogenized at 0°C in 5 volumes of 0.05 M Tris-HCl buffer, pH 7.5, using a glass Potter-Elvehjem homogenizer. Turbo hepatopancreas was minced and defatted with cold acetone (–15°C), because its high mucosity. The air-dried acetone powder of turbo hepatopancreas was homogenized with 20 volumes of 0.05 M Tris-HCl buffer, pH 7.5. Hepatopancreas homogenate from whelk, turbo, and squid were centrifuged at 4°C as outlined previously (6). After differential centrifugation, the 105,000g supernatant was heated at 70°C for 10 min in the presence of 10 mM 2-mercaptoethanol. The isolation and purification of cadmium-binding proteins have been fully described (6).

Electrophoretic characterization of the purified Cd-binding proteins was carried out on a standard pH 8.9 polyacrylamide disc gel system as described by Williams and Reisfeld (7) and by Weber and Osborn (8) in the case of SDS–polyacrylamide gel electrophoresis. Isoelectric focusing was carried out with carrier ampholytes in the pH range of 3.5 to 10.0 in a small column (50 mL) as described earlier (6,8).

Cadmium and copper concentrations were determined by flame or flameless atomic absorption spectrophotometry, and mercury concentration was obtained by cold vapor atomic absorption spectrophotometry.

Table 1. Metal content in the hepatopancreas of whelk, turbo, and squid.

| Species | N<sup>ab</sup> | Cd   | Zn   | Cu   | Ca   |
|---------|---------------|------|------|------|------|
| Whelk   | 110           | 119.4 ± 83.1 | 192.3 ± 124.2 | 211.7 ± 156.1 | 567.7 ± 150.8 |
| Turbo   | 75            | 33.2 ± 10.9 | 62.2 ± 13.6 | 6.1 ± 1.4 | 632.7 ± 185.2 |
| Squid   | 90            | 50.4 ± 26.1 | 78.4 ± 68.4 | 157.2 ± 126.5 | 45.2 ± 20.7 |

*The range of metal concentrations in individual tissues are shown in parentheses.

The protein and sugar content was determined by the method of Nakao et al. (10) and by the phenol–sulfuric acid method (11), respectively.

Amino acids were analyzed on an automatic amino acid analyzer after oxidation with performic acid and hydrolysis with 6 M HCl at 110°C in evacuated sealed tubes for 20 and 48 hr according to the method of Moore (12). Amino sugar was also determined after hydrolysis with 4 M HCl at 100°C for 8 hr. Carbohydrate composition was determined by gas-liquid chromatography as described previously (6).

The assay of metal binding to Cd-binding glycoproteins was carried out with equilibrium dialysis at 4°C against 0.05 M Tris-HCl buffer, pH 7.0, containing from 1.0 to 200 μM CdCl<sub>2</sub>, CuCl<sub>2</sub>, or HgCl<sub>2</sub> for 17 hr.

Results and Discussion

In general, heavy metals such as cadmium are concentrated to a greater extent in molluscs than in other species of mollusc hepatopancreas.

Table 2. Percentage of total cellular metals in cytosol of three species of mollusc hepatopancreas.<sup>a</sup>

| Species | Cd, % | Zn, % | Cu, % | Ca, % |
|---------|-------|-------|-------|-------|
| Whelk   | 30    | 47    | 25    | 61    |
| Turbo   | 11    | 46    | 51    | 57    |
| Squid   | 43    | 50    | 66    | ---b |

<sup>a</sup> Percent recovered in 105,000g supernatant.

<sup>b</sup> Not determined.
phyla. Heavy metal concentrations in the hepatopancreas of these molluscs, particularly in the whelk, conspicuously exceed those in all other organs in spite of a trace amount of cadmium in the seawater (0.02 μg/L). As shown in Table 1, the concentration of cadmium varies from 47 to 350 μg/g wet tissue for whelk, 15 to 50 μg/g wet tissue for turbo, and 10 to 95 μg/g wet tissue for squid. Cadmium, zinc, and copper were predominantly enriched in these hepatopancreas, but copper in turbo hepatopancreas was very low. This may be due to its being planktotrophic. Two shellfish hepatopancreas contained high concentration of calcium. The three species of mollusces analyzed had no known exposure to levels of metals other than environmental concentrations.

By differential centrifugation of whelk, turbo, and squid hepatopancreas homogenates, 30%, 11%, and 48% of cadmium respectively, in each hepatopancreas was extracted to the soluble fraction (Table 2), in contrast with cadmium distribution in mammalian liver in which most of the cadmium was recovered in the cytosol.

Gel-permeation chromatography on a calibrated Sephadex G-75 column was performed on each 105,000g supernatant of hepatopancreas homogenates from three species of mollusces in the presence of 5 mM 2-mercaptoethanol. In each profile, cadmium is almost exclusively associated with a low molecular weight fraction eluting in the same position regardless of species. Each mollusc supernatant was found to exhibit a different elution profile with respect to the Cd-binding peak, whether in the absence or presence of 2-mercaptoethanol (Fig. 1). Heat treatment and extraction at pH 7.5 was effective in isolation of heat-stable, relatively low molecular weight proteins from tissues and in preventing attack by contaminating glycosidases which are predominant in the hepatopancreas of gastropods.

For further purification of the main Cd-containing fraction, termed FII, chromatography on a DEAE–Sephadex A-25 column was performed with the result shown in Figure 2. By fractionation with a salt linear gradient on a DEAE–Sephadex A-25 column, the Cd-containing fraction was separated into at least two species for either the whelk or the turbo hepatopancreas. In the case of the squid, only one Cd-binding protein was isolated with pH gradient elution.

Two unique Cd-binding glycoproteins from whelk, Buccinum tenuissimum, hepatopancreas were purified to homogeneity by chromatography on a second DEAE–Sephadex A-25 column and isoelectric focusing. Figure 3A shows a rechromatogram on DEAE–Sephadex A-25 of the partially purified Cd-binding protein from the first DEAE–Sephadex A-25 column. By use of a shallower linear gradient, two main cadmium peaks coinciding with the protein peaks could be separated completely. For convenience, the two elution peaks are referred to as FII_A and FII_B in elution order. Each component showed a single band on disc electrophoresis as described later.

The crude Cd-binding fraction, FII, was also subjected to electric focusing at pH 3.5 to 10.0 by using a minicolumn and shown to have isoelectric points, 6.57 and 6.05 of subforms from FII_A, and pI 5.08 and 4.77 of those from FII_B, as determined by disc electrophoresis (Fig. 3B). This polymorphism may be due to their having a different content of cadmium, since it is readily dissociable.

![Figure 2](image1.png)  
**FIGURE 2.** DEAE–Sephadex A-25 column chromatography of Cd-containing fraction FII from Sephadex G-75 gel filtration: (●), Cd concentration; (→), absorbance at 280 nm; (---) NaCl concentration or pH.

![Figure 3](image2.png)  
**FIGURE 3.** Purification of Cd-binding glycoproteins from whelk hepatopancreas: (●) Cd concentration; (×), Cu concentration; (→) absorbance at 280 nm; (---) NaCl concentration. (A) Rechromatography on second DEAE–Sephadex A-25 of the partially purified Cd-binding glycoprotein from the first DEAE–Sephadex A-25 column. (B) Isoelectric focusing of Cd-binding glycoproteins FII_A and FII_B. Each fraction was 0.7 mL. The dots represent pH measured at 4°C.
Starting from 50 g of whelk hepatopancreas, about 13.5 mg and 21.5 mg purified Cd-binding proteins FIIA and FIIB, respectively, were obtained, representing a yield of approximately 0.07% on a wet weight basis and containing about 0.6% and 1.0% cadmium by weight, respectively.

The two purified Cd-binding proteins, FIIA and FIIB, from whelk hepatopancreas were fairly homogeneous, as shown by disc electrophoresis at pH 8.9 on 10% polyacrylamide gel. Figure 4A shows the Coomassie blue-stained protein bands. Gels were sliced in 0.3 cm thickness and cadmium content of each segment was measured by the flameless atomic absorption spectrophotometer. As can be seen in Figure 4B, the apparent cadmium was detected in the gel section, corresponding to that stained as protein. Each of the purified fraction gave a faint positive periodic acid–Schiff band. This suggests that these Cd-binding components are glycoproteins.

On SDS–polyacrylamide gel electrophoresis, FIIA and FIIB also showed a single band each, with apparent molecular weights of 8000 and 18,000, respectively, which were obtained by calibration of molecular weight by using standard proteins. FIIA was found to be stable for at least 72 hr in the absence or presence of 0.5% 2-mercaptoethanol at room temperature. On the other hand, FIIB was found to dissociate gradually to a molecular weight of about 8000 in the same concentration of SDS and 2-mercaptoethanol (θ). This suggests that FIIB consists of two polypeptide chains having different molecular sizes linked with the disulfide bond.

Amino acid and carbohydrate analysis were performed on FIIA and FIIB. These components consisted of about 79.7% protein and 20.3% sugar for the former and 91.3% protein and 8.7% sugar for the latter (by weight percentage), as shown in Table 3. The metal-free minimum molecular weight of the protein moiety was calculated from the amino acid composition yielding values of 6570 for FIIA and 11,910 for FIIB, which agree well with the values determined by SDS–polyacrylamide gel electrophoresis.

Table 3. Amino acid and carbohydrate composition of purified cadmium-binding glycoproteins from whelk hepatopancreas.

| Mole/mole glycoprotein | FIIA  | FIIB  |
|------------------------|-------|-------|
| Amino acid (weight %)  |       |       |
| Aspartic acid          | 5.13  | 8.86  |
| Threonine              | 4.66  | 11.09 |
| Serine                 | 3.91  | 6.86  |
| Glutamic acid          | 4.00  | 6.15  |
| Proline                | 2.97  | 4.11  |
| Glycine                | 4.31  | 8.47  |
| Alanine                | 4.23  | 7.53  |
| Half-cystine           | 2.56  | 3.72  |
| Valine                 | 3.12  | 5.91  |
| Methionine             | 0.74  | 1.24  |
| Isoleucine             | 2.27  | 4.18  |
| Leucine                | 3.09  | 5.11  |
| Tyrosine               | 0.83  | 3.41  |
| Phenylalanine          | 1.88  | 4.71  |
| Tryptophan             | 2.89  | 3.06  |
| Lysine                 | 3.17  | 6.06  |
| Histidine              | 1.18  | 6.98  |
| Arginine               | 1.90  | 2.79  |
| Total residues         | 52    | 94    |
| Carbohydrate (weight %)|       |       |
| Glucosamine            | 0.88  | 6.55  |
| Galactosamine          | -- (--) | 1.10 (1) |
| Fucose                 | 1.11 (1) | 1.82 (2) |
| Mannose                | 1.62 (2) | 1.94 (2) |
| Glucose                | 0.75 (1) | 0.71 (1) |
| Arabinose              | 1.92 (2) | -- (--) |
| Galactose              | 3.13 (3) | 2.04 (2) |

* Average of 20 and 48 hr hydrolyses unless indicated otherwise.

O Obtained by extrapolation to zero hydrolysate time.

Cys and Met were determined as cysteic acid and methionine sulfone, respectively.

d Estimated spectrophotometrically.

e Determined with the amino acid analyzer after hydrolysis.

f Denotes 0.1 mole/mole or less.
phoresis. In the N-terminal amino acid analysis by dansylation method (15), lysine alone was found for FIIA and lysine and glycine for FIIB. The numbers of main sugar components were similar to each other in FIIA and FIIB. Three and two free sulfhydryl groups in FIIA and FIIB, respectively, were detected by the titration with p-chloromercuribenzoate (14), and after performic acid oxidation two more half-cystine were found in FIIB.

Cd binding or Cu binding of the two glycoproteins was measured by the equilibrium dialysis method. In Figure 5, the Cd$^{2+}$ bound by FIIA or FIIB is plotted against molar concentration of free Cd$^{2+}$. Solid lines are Cd saturation curves after treatment with 2-mercaptoethanol, and dotted lines are those without treatment. The non-equivalence of the dissociation constants in the presence or absence of 2-mercaptoethanol might suggest the glycoproteins were not easily accessible for Cd$^{2+}$ because of autoxidation of sulfhydryl groups. The binding data were analyzed with the Scatchard equation to obtain the dissociation constant, $K_d$, and the maximum number of metal ions bound. The straight line has a slope equal to $1/K_d$, and the intercept on the abscissa is equal to the maximum number of metal ions bound, $n$. From Figure 6, the apparent dissociation constants and maximum number of Cd$^{2+}$ bound were $K_d = 7.3 \times 10^{-7}$M, $n = 6$ for FIIA and $K_d = 9.1 \times 10^{-7}$M, $n = 8$ for FIIB. In the case of FIIA, the Scatchard plot shows convexity. This probably indicates the presence of some cooperativity in the Cd binding.

Beside cadmium enrichment, the copper concentration rises throughout purification to 6 $\mu$g/mg protein. The two glycoproteins have high affinity also for copper after reduction with 2-mercaptoethanol. The maximum number of Cd and Cu ions bound appeared to be non-equivalent, and dissociation constants for copper were somewhat larger than for Cd (6). At saturation of Cd, the amount of Cu firmly bound to the glycoprotein did not decrease, and this suggests that Cd and Cu ions may both interact with the independent binding site, not replacing one another.

The affinity of the glycoproteins for mercury was also very high, and the binding was stoichiometric between titrable sulfhydryl groups and bound Hg$^{2+}$ as shown in Figure 7.

In saturation binding with Cd, the whelk Cd-binding glycoprotein showed kinetic characteristics markedly different from those of metallothionein, in which metals ligate to the 20 metal-coordinating cysteine residues, to form the Cd-cluster (15). Here metallothionein has no disulfide bond nor any free sulfhydryl groups. In contrast, the binding of whelk Cd-binding glycoprotein was a reversible saturable process with high affinity for free Cd$^{2+}$ and Cu$^{2+}$ ions, not for Zn$^{2+}$ ion. The low content of cysteine is not sufficient as an explanation of the binding of the sum of Cd$^{2+}$ and Cu$^{2+}$ present. In order to
understand the role of sulfhydryl groups on metal binding, Cd binding was measured in the presence of Hg2++. Figure 8 shows that FII2 could bind Cd2+ even though under such free Hg2+ concentration as SH groups were blocked with Hg2++. This suggests that sulfhydryl groups in the glycoprotein are necessary not for metal coordination, but for keeping high affinity conformation for cadmium.

One possible explanation for metal binding sites might be the importance of Cd-binding pockets, which, in association with the oligosaccharide, can determine specificity of the metal-binding glycoprotein. Furthermore, the partially purified Cd-binding component from turbo hepatopancreas by our procedure contained 25 to 45% carbohydrate. Clubb et al. (16) have reported Cd-containing glycoprotein from stonefly, *Petronarcys californica*, to be enveloped by the carbohydrate. This glycoprotein bound cadmium in spite of larger content of sugar than that of protein.

In summary, based on its common properties, namely, (1) binding specificity for Cd and mercury, (2) amino acid composition, and (3) molecular weight, the Cd-binding glycoprotein of the hepatopancreas was not identified as a metallothionein, but as a new Cd-binding protein.

Comparison between mammalian metallothionein and the Cd-binding glycoproteins from whelk hepatopancreas (Table 4) shows the similarities to be low molecular weight, polymorphism, and acidic protein, and differences to be the content of cysteine residues (20 moles versus 3 or 2 moles per mole protein), serine and lysine content, which may have an important role in heavy metal complexing with cysteines (17), N-terminal amino acid, and sugar content of these two kinds of protein.

These suggest differences in their biophysiological roles and in the availability of heavy metals in the environment between these two kinds of protein. Whelk may be a relatively insensitive species to cadmium toxicity.

From the amino acid composition, Cd-binding glycoproteins from whelk hepatopancreas are similar to Cd-binding protein from rat bone (18), rather than to hepatic metallothionein (19). It is interesting, in relation to calcium transport, that Cd-binding protein with high affinity for cadmium or copper exists in such calcium-rich tissues as shellfish hepatopancreas or mammalian bone.

It can be assumed that Cd-binding glycoprotein exists in the tissue as a normal constituent without metals, and becomes a site for the accumulation of cadmium or copper because of its Cd-chelating ability.

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### Table 4. Comparison of molecular properties between whelk cadmium-binding glycoproteins and mammalian metallothioneins.

| Characteristic                  | Whelk Cd-binding GP* | Metallothionein* |
|--------------------------------|----------------------|------------------|
| Metal specificity              | Cd, Cu, Hg           | Cd, Zn, Cu, Hg   |
| Maximum metal bound            | 6 mole, 8 mole       | 6–7 mole        |
| Molecular weight               | 8000, 13,000         | 6000–7000       |
| Isoelectric point (pI)         | FII2, FII4          | MT-I, MT-II     |
| Chemical composition           |                      |                 |
| Protein content, %             | 79.7, 91.3           | 100             |
| Carbohydrate content, %        | 20.5, 8.7            | —               |
| Amino acid composition         |                      |                 |
| Total residues                 | 52, 94               | 61, 61          |
| Predominant amino acid, cysteine| 3, 2                 | 20, 20          |
| N-Terminal amino acid          | Thr, Asp, Ser, Gly  | Lys, Ser, Gly   |
|                                | Lys, Gly             | N-Acetyl Met    |

* Abbreviation of glycoprotein.
* From the literature data (17,19–22).

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