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Isolation and Partial Characterization of Cadmium Binding Proteins from the Oceanic Squid, *Ommastrephes bartrami**

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Abstract

Cadmium binding proteins were isolated from the liver of the squid, *Ommastrephes bartrami*, collected from the North Pacific Ocean. Based on the elution position on Sephadex G-75, its apparent molecular weight is about 16,000. By ion exchange chromatography, the cadmium-protein complexes were separated further into a cadmium binding protein and two cadmium, copper binding proteins. Each metal binding protein contains about 21-29% dicarboxylic acids (Aspartic and Glutamic acids), traces of histidine and phenylalanine, and a proportionally higher glycine content than horse metallothionein. Based on the amino acid composition, it seems that the cadmium binding proteins from the squid are different from the mammalian metallothionein.

Introduction

Accumulation of heavy metals by several organisms has been shown to be associated with the production of special proteins that can bind and sequester, toxic and excess heavy metals. The first of these heavy metal binding proteins was isolated from the horse kidney by Margoshes and Vallee (1957). It was a cadmium binding protein and was subsequently called metallothionein because of its strong affinity for heavy metals and high cysteine content (25-30%). Aside from these properties, metallothioneins are characterized as (Kägi and Kojima, 1987): 1) low molecular weight proteins, about 6,000-7,000, although on gel permeation chromatography it behaves like a 10,000 spherical protein; 2) contain no aromatic amino acids and histidine; and 3) have maximum absorbance at about 250 nm, a characteristic of metal thiolates.

In several organisms, such as: freshwater clam (Doherty et al., 1987); mussels (Nöel-Lambot, 1976; Frankenne et al., 1980; Roesijadi and Unger, 1988; Viarengo et al. 1987); snails (Langston and Zhou, 1986; Dallinger et al., 1989) and crabs (Overnell and Trewhella, 1979; Ridlington et al., 1981; Lerch et al., 1982; Otvos et al. 1982; Overnell, 1984a & b), metallothionein-like heavy metal binding proteins have been isolated and identified. On the other hand, heavy metal binding proteins having characteristics different from horse metallothionein have also been reported for the crayfish (Lyon et al., 1983), whelk (Dohi et al., 1983) and scallop (Stone and Overnell, 1985).

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Considering that the cadmium concentration can be as high as 211 μg/g wet weight in the liver of the squid, *Ommastrephes bartrami* (Castillo and Maita, in prep.), it seems vital that these squids have a mechanism of detoxifying and storing heavy metals in a non-toxic and/or non-reactive form. In this study, we isolated and purified cadmium binding proteins from the liver of *O. bartrami* and determined its amino acid composition.

Materials and Methods

Animal samples

Squid samples were collected by jigging in the western North Pacific Ocean (36.5°N, 155°E) in June, 1989. The samples were wrapped separately and frozen (−20°C) immediately after collection.

Preparation of liver extract

In the laboratory, a 63 g liver was homogenized in 20 mM Tris-HCl buffer, pH 8.5, containing 5 mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000×g for 30 min and the supernatant heated at 70°C for 5 min. Heat labile compounds were removed by centrifugation at 20,000×g for 30 min. To the supernatant, prechilled acetone was added dropwise to about 60-70% by volume to precipitate heat stable proteins, which were collected by centrifugation at 20,000×g for 10 min. The precipitate was resuspended in Tris-HCl buffer and ultracentrifuged at 105,000×g for 90 min. Unless otherwise stated, the above steps were performed at 4°C.

Chromatographic purification

The supernatant after ultracentrifugation was applied to a Sephadex G-75 column (2.6×100 cm) which has been equilibrated with the Tris-HCl buffer. Elution was done at a flow rate of 30 ml·h^{-1} and 5 ml fractions were collected. Blue dextran, bovine serum albumin (66 K), α-chymotrypsinogen (24.5 K), cytochrome c (12.4 K) and insulin B (3.5 K) were used to standardize the Sephadex column. The optical density of each fraction was monitored at 280 and 254 nm with a Hitachi spectrophotometer, Model 200-20. Since heavy metal binding proteins usually bind more than one kind of metal, the cadmium, copper and zinc concentrations were determined in the fractions, by direct aspiration into a Nippon Jarrel Ash atomic absorption spectrophotometer, Model AA-782.

Cadmium rich fractions indicated by the bar in Fig. 1 were pooled and rechromatographed on a Sephadex G-50 column (1.7×90 cm). The second cadmium peak in Fig. 4 (Fr. II) was applied to a 1.6×5.0 cm ion exchange column (Sephadex A-25). Elution was done step-wise by increasing the NaCl concentration in the buffer from 0.1 to 0.5 M. The optical density and heavy metal concentrations were monitored as before.

Amino acid analysis

Fraction II (Fig. 4) and heavy metal rich fractions (Fig. 5) after ion exchange chromatography were oxidized with performic acid and hydrolyzed in 6 N HCl at 110°C for 24 h. The amino acid composition of each sample was determined using

a Shimadzu High Performance Liquid Chromatograph, Model LC-5A. The amino acid standard solution, type H (Wako Pure Chemical Industries, Japan), was injected several times giving a coefficient of variation of $7 \pm 2\%$ for all the amino acids determined.

Results and Discussion

Fractionation of the heat stable precipitate by gel permeation chromatography on Sephadex G-75 revealed that the cytosolic cadmium, copper and zinc are bound to different proteins, as shown by the elution profiles of the heavy metals (Fig. 1). Although there are positions where the peaks or shoulders of the three heavy metals coincide, the major peaks were observed at different positions.

The molecular weights of the metal-prtotein complexes were estimated based on the elution positions of standard proteins on Sephadex G-75 (Fig. 2). The main cadmium peak was observed at the 16 K position and shoulders at the 30 K and 10 K positions. On the other hand, the main copper peaks were observed at the 43 K with a shoulder at the 30 K and at the 10 K positions, and a small peak at the 16 K positions. The main zinc peak was found at the 9 K position and two smaller peaks at the 30 K and 16 K positions. This is in contrast to that observed for *Onychoteuthis borealijaponica* (Castillo et al., 1990) and *Todarodes pacificus* (Tanaka et al., 1983), where the main cadmium peaks were observed at the low ($M_r < 3$ K) and high molecular weight ($M_r > 70$ K) positions, respectively. Although in both cases the elution buffer used during gel chromatography did not contain 2-mercaptoethanol, the liver extract from *O. borealijaponica* was prepared with Tris-HCl buffer contain-

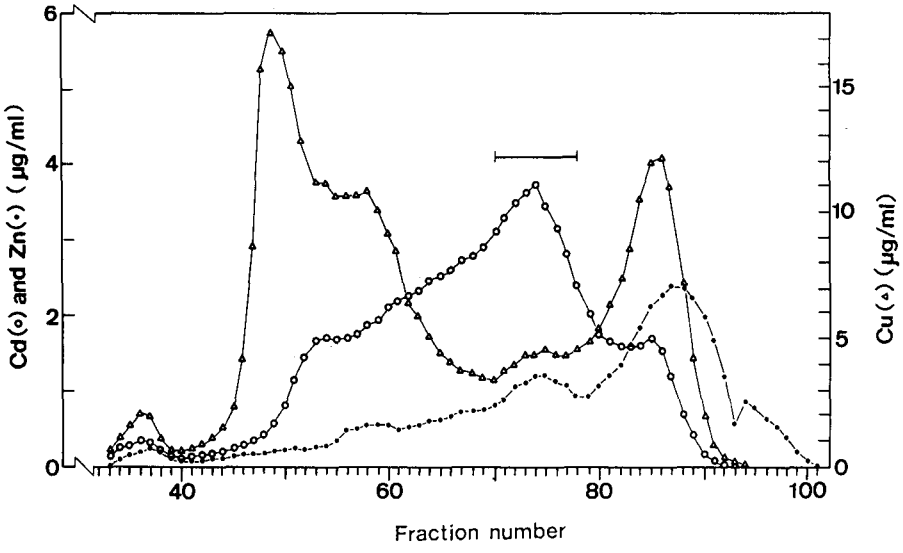

Fig. 1. Heavy metal concentrations in the fractions separated by permeation chromatography on Sephadex G-75 of the liver cytosol from the squid, *Ommastrephes bartrami*. Elution was done using Tris-HCl buffer, pH 8.5, containing 5 mM 2-mercaptoethanol. The bar indicates the fractions which were pooled for rechromatography.

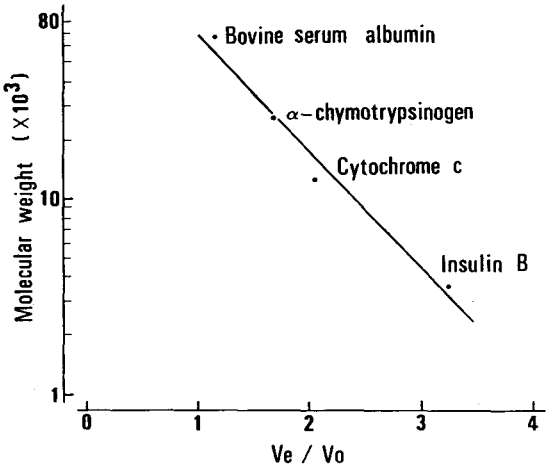

Fig. 2. Estimation of apparent molecular weight of heavy metal binding proteins by permeation chromatography on Sephadex G-75 using several proteins whose molecular weights are well-known.

ing 5 mM mercaptoethanol. This may explain why the elution positions of the main cadmium binding compounds were different for *O. borealijaponica* and *T. pacificus*.

To test whether the binding of the heavy metals is affected by the presence of 2-mercapoethanol, a second purification was done in the absence of 2-mercaptoethanol. Figure 3 shows that the elution profiles of cadmium, copper and zinc in the absence of 2-mercaptoethanol are different. Cadmium and zinc were eluted mainly at the void volume position ($M_r > 70$ K) whereas copper was eluted mainly at the low molecular weight position ($M_r < 3$ K), similar to the elution profile

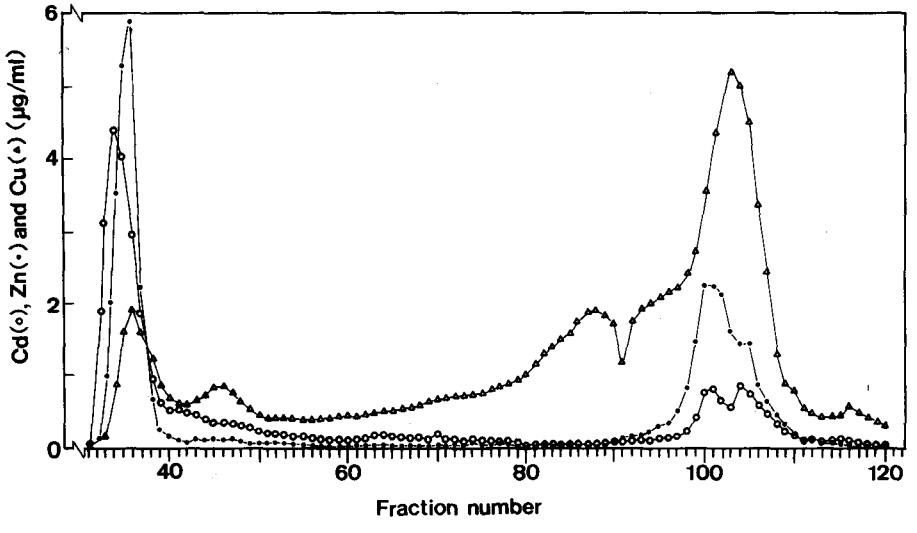

Fig. 3. Elution profile on Sephadex G-75 of the liver cytosol from the squid, *Ommastrephes bartrami*, in the absence of 2-mercaptoethanol.

observed by Tanaka et al. (1983).

Comparing Figs. 1 and 3, it is apparent that 2-mercaptoethanol has a considerable effect on the binding of the heavy metals. Since the physiological reducing level in the hepatocytes is around 5 mM (Jocelyn, 1973, Cited in Hidalgo et al., 1988), it is possible that the addition of 5 mM 2-mercaptoethanol in the buffer used created a condition similar to that present in the hepatocytes. Hence, the elution position of the proteins on Sephadex G-75 in the presence of 2-mercaptoethanol can be considered as approximating that of the native state. In the absence of a reducing agent, it is possible that during the preparative stages polymerization occurred, explaining the major occurrence of high molecular weight, metal-protein complexes which were eluted in the void volume position (Fig. 3). Considering that the cysteine content of the heavy metal binding proteins is very low (Table 1), it appears that 2-mercaptoethanol only provides a reducing environment similar to that existing in the body and does not reduce the SH bonds of the protein, a function associated with 2-mercaptoethanol.

Since our interest is in isolating the cadmium binding proteins, the main cadmium peak from Sephadex G-75 (indicated by the bar in Fig. 1) was rechromatographed on Sephadex G-50. Again-the metal-protein complexes were separated into three cadmium peaks, two copper peaks, and two zinc peaks (Fig. 4). To separate the different charge forms of the metal-protein complexes present in the second cadmium peak, Fr. II (Fig. 4) was applied to an ion exchange column. Although all the metal-protein complexes were eluted at 0.1 M NaCl, it can be seen in Fig. 5 that the elution positions of the metal-protein complexes were different. Zinc was eluted

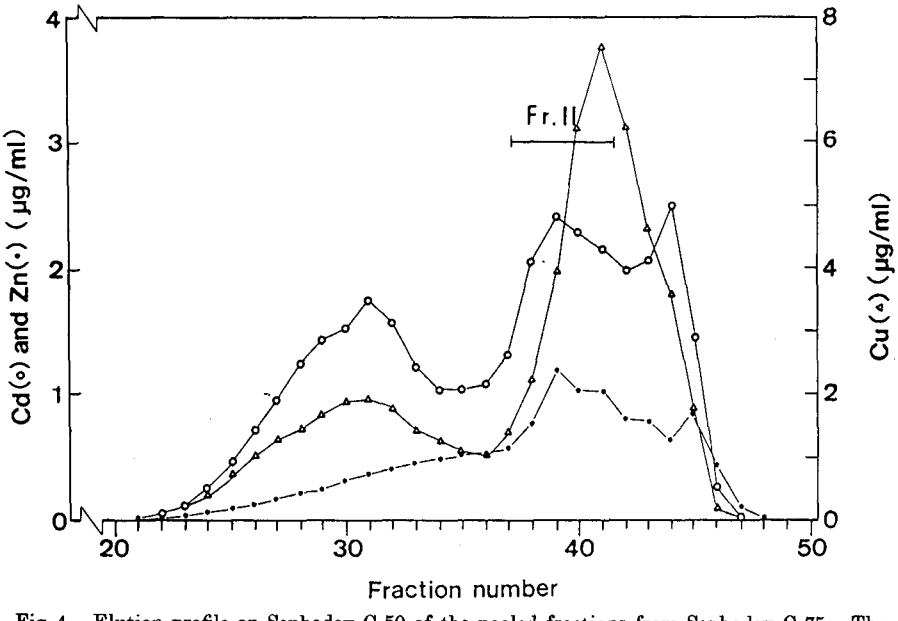

Fig. 4. Elution profile on Sephadex G-50 of the pooled fractions from Sephadex G-75. The bar indicates the fractions which were pooled for further purification by ion exchange chromatogaraphy.

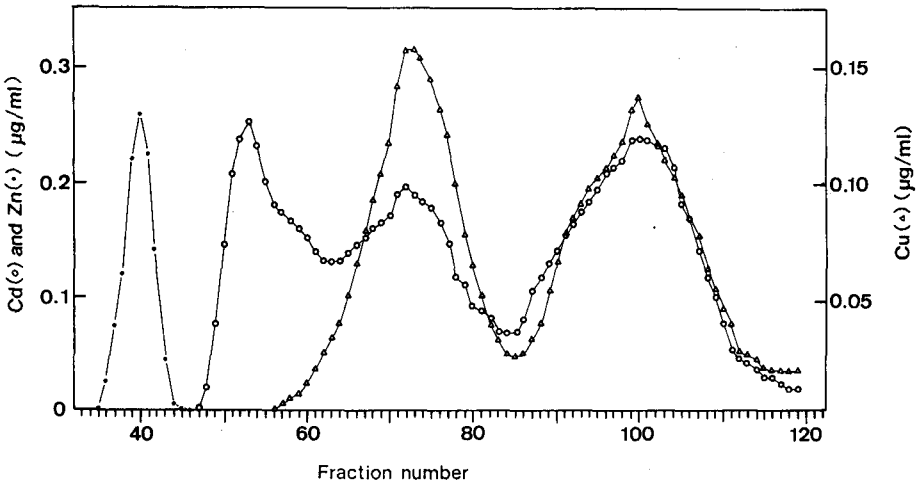

Fig. 5. Ion exchange chromatogram of Fraction II on DEAE Sephadex A-25. Almost all of the metals were eluted at 0.1 M NaCl in Tris-HCl buffer, pH 8.5, containing 5 mM 2-mercaptoethanol.

Table 1. Amino acid compositions (mol %) of heavy metal binding proteins isolated from the squid, *Ommastrephes bartrami*, collected from the western North Pacific Ocean in 1989.

Amino Acid	SG-50 Fr. II	Ion Exchange Chromatography			
		Zn-BP	Cd-BP	Cd, Cu-BP	Cd, Cu-BP
1/2 Cys[1]	0.7	nd[2]	nd	nd	nd
Asp	13.5	13.6	15.8	12.3	9.7
Thr	7.7	6.6	6.6	6.5	5.0
Ser	10.2	11.7	15.4	10.4	15.1
Glu	8.3	9.9	12.8	9.4	11.4
Pro	6.0	5.1	t	5.9	3.5
Gly	13.3	14.8	21.4	16.2	25.7
Ala	9.0	9.4	10.7	9.4	8.0
Val	8.1	7.1	t[3]	7.7	4.2
Met	nd	nd	nd	nd	nd
Ile	6.8	6.3	7.0	6.6	5.3
Leu	8.4	10.1	10.3	10.4	10.7
Tyr	nd	nd	nd	nd	nd
Phe	2.7	3.3	nd	3.4	nd
His	0.5	1.1	t	t	1.6
Lys	2.0	1.0	nd	1.7	nd
Arg	2.8	nd	nd	nd	nd

[1] determined as cysteic acid
[2] not detected
[3] trace amounts

first, followed by cadmium, and then two peaks containing both cadmium and copper. Since separation based on the molecular weight was done twice, it is assumed that the metal rich fraction after ion exchange chromatography was relatively pure and is already acceptable for amino acid analysis. Turanek et al. (1987) observed that the rat liver Cd, Zn-metallothionein can be rapidly purified by almost the same preparative steps: heat treatment at 60°C; precipitation by acetone; and desalting on Sephadex G-25.

Although the optical density was monitored at 280 and 254 nm, the absorption profiles were not included since the separation of the heavy metal binding proteins is better elucidated by the elution profiles of the heavy metals.

Amino acid composition of the fractions indicated by the bar in Fig. 4 and the heavy metal peaks in Fig. 5 are shown in Table 1. The intermediate molecular weight fractions after chromatography on Sephadex G-50 (SG-50 in Table 1) showed very low cysteine contents, and after ion exchange chromatography, cysteine was no longer detected. Considering that cysteine was not a major amino acid, it is

Table 2. Amino acid compositions (mol %) of Molluscan cadmium binding proteins with high glycine contents.

Amino Acid	Horse[1] liver	Mussel[2] CdBP 20		Mussel[3]	Oyster[4]	Squid[5]		
		20I	20III			Cd-BP	Cd, Cu-BP	Cd, Cu-BP
1/2 Cys[6]	32.6	26.1	18.2	25.5	24.8	nd[7]	nd	nd
Asp	5.0	7.9	13.3	8.1	6.8	15.8	12.3	9.7
Thr	3.9	7.5	9.2	6.4	6.9	6.6	6.5	5.0
Ser	11.6	7.9	7.2	7.9	9.4	15.4	10.4	15.1
Glu	4.5	4.1	8.0	4.5	6.4	12.8	9.4	11.4
Pro	5.1	4.8	3.4	6.8	5.5	t	5.9	3.5
Gly	10.0	18.2	14.8	15.8	15.7	21.4	16.2	25.7
Ala	9.4	3.8	10.3	5.2	5.9	10.7	9.4	8.0
Val	2.6	4.8	5.1	3.8	2.3	t	7.7	4.2
Met	1.5			<0.5	0.7	nd	nd	nd
Ile	0.6	3.0	3.1	4.0	1.5	7.0	6.6	5.3
Leu	0.6	0.5	3.1	1.3	1.0	10.3	10.4	10.7
Tyr				<0.5	0.6	nd	nd	nd
Phe				1.3	0.5	nd	3.4	nd
His		t[8]				t	t	1.6
Lys	10.4	10.0	4.6	7.7	11.3	nd	1.7	nd
Arg	2.2	1.5	nd	1.8	0.6	nd	nd	nd

[1] Kagi et al. (1974)
[2] Frazier et al. (1985)
[3] Frankenne et al. (1980)
[4] Frazier and Brouwer (In Frazier et al., 1985)
[5] this study
[6] determined as cysteic acid
[7] not detected
[8] trace amounts

possible that the heavy metals are bound to the dicarboxylic amino acids, aspartic and glutamic acids, instead of the usual binding to cysteine as in the metallothionein present in other invertebrates. Considering that metallothionein contains about 25-30% cysteine, it is interesting that the combined molar ratios of aspartic and glutamic acids are 24%, 29% and 21-22% for Zn-BP and Cd-BP, and Cd, Cu-BP, respectively.

In Table 2, the amino acid composition of cadmium binding proteins from different molluscs were compared to that of the squid. Although the cysteine contents of the heavy metal binding proteins from the squid were markedly different, all the molluscs shown have a higher glycine content than horse metallothionein. Frazier et al. (1985) suggested that the higher glycine content observed in the mussel and oyster may indicate the presence of a unique class of metallothionein-like proteins in molluscs, which are characterized by a lower cysteine content and a relatively higher glycine content. Furthermore, he noted that a higher glycine content may imply an evolutional advantage of glycine in aquatic invertebrate cadmium binding proteins. It should be noted, however, that not all of the cadmium binding proteins isolated from molluscs have a high glycine content, as in whelk (Dohi et al., 1983) and snail (Dallinger et al., 1989).

Our results show that the liver of the squid contains cadmium binding proteins that are different from the metallothionein found in mammals. Further studies are needed to clarify the biochemical nature of these proteins, such as the metal binding sites and the role of these substances in the detoxification process.

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