

Occurrence of Cadmium in Crabs (*Cancer pagurus*) and the Isolation and Properties of Cadmium Metallothionein

by Julian Overnell*

The occurrence of high cadmium levels in the digestive gland (hepatopancreas) of the crab *Cancer pagurus* is reviewed and its possible significance to human health is discussed. Previous attempts to isolate this cadmium in its native form are briefly reviewed and the present method used in this laboratory to isolate crab cadmium metallothionein is described. Quantitative amino acid analysis of the purified protein suggests that it has a total of 55 residues, including 19 Cys, and a molecular weight of approximately 5800. This yields an extinction coefficient at 220 nm for the protein in HCl, pH 1.5, of 50,000 and at 250 nm for the native cadmium protein in Tris-HCl, pH 8.4, of 92,000.

Introduction

The natural occurrence of high levels of cadmium in the edible crab *Cancer pagurus* is now well substantiated. The first report of elevated cadmium levels (up to 10 ppm wet weight) in crab meat (1) stimulated a number of investigations. Topping (2) (1973) found values of up to 13 ppm wet weight for the gonad plus liver of crabs from Shetland, and Portmann (3), in a systematic study of crabs from English coastal waters found values of cadmium in the body meat (mostly digestive gland) of up to 70 ppm. Overnell and Trehwella (4) examined crabs from Orkney and Shetland and found values of cadmium of up to 50 ppm wet weight in the digestive gland. This study (Fig. 1) showed that, although most animals have values of cadmium between 5 and 20 ppm, a few individuals have much higher values. A 40 g hepatopancreas from a crab containing 50 ppm Cd in that organ contains 2 mg of cadmium. The FAO/WHO provisional tolerable weekly intake of cadmium is 400 to 500 μ g. The actual uptake of cadmium from food may be influenced by many factors (5), including the calcium levels in the diet and high levels of calcium phosphate are present in crab digestive glands. In a recent report, Newton et al. (6) found that cadmium in crab sandwiches, made from crabs fed with 115m Cd after 10 days incubation, was retained by seven human volunteers to the extent of approximately 1.2 to 7.6%. This is a similar value to that found by other workers with extrinsically labeled food (6). Results of feeding studies in which rat liver metallothionein was fed to mice indicated that metallothionein cadmium was transported preferentially to the kidney rather than to the liver (7).

In contrast, inorganic cadmium is transported preferentially to the liver (7). Since naturally occurring cadmium in high-cadmium crabs is present entirely bound to metallothionein (which is heat-stable), it is possible that this cadmium may also be preferentially accumulated in human kidneys when the animals are consumed.

It was shown that cadmium in the diet of crabs, in the form of cadmium-treated mussels, was readily absorbed and retained by the digestive gland (4). Davies et al. (8) have presented evidence that the distribution of cadmium between the gonad, gill, and hepatopancreas of Orkney crabs is consistent with uptake of cadmium into the animals from the diet, but inconsistent with uptake from water. The nature of the presumed high cadmium component(s) present in the diet of Orkney crabs is not yet known but is probably correlated with geochemical factors (9).

Earlier studies in this laboratory indicated that cadmium present both in wild high-cadmium crabs and crabs after injection of cadmium (followed by a 4-week incubation period) was present mostly in the hepatopancreas. Essentially all the cadmium injected into low-cadmium animals could be recovered in the hepatopancreas after periods of up to 6 months. The cadmium concentration of the hepatopancreas was much higher than that in any other organ, and this organ is also the largest in the body. The cadmium concentration in the high speed supernatant of a homogenate of hepatopancreas is always as great as that in the original homogenate itself, indicating that all the cadmium is soluble. Figure 2, redrawn from Overnell, (10), indicates that all the soluble cadmium is present in a single peak during chromatography on Sephadex G-75. This peak corresponds to the elution position for metallothionein. The result is the same for crabs

*NERC Institute of Marine Biochemistry, St. Fittick's Road, Aberdeen AB1 3RA, United Kingdom.

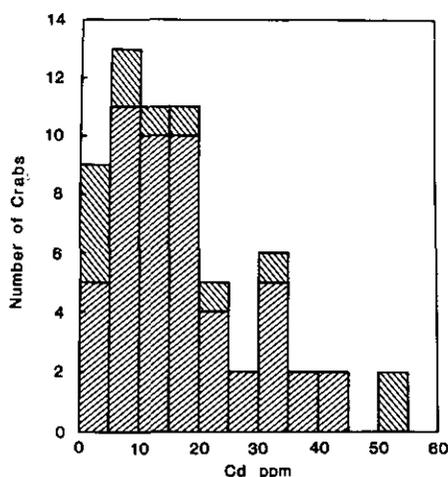


FIGURE 1. Distribution of cadmium concentration (ppm wet weight) in the hepatopancreas of 63 specimens of crab (*Cancer pagurus*) from (▨) Orkney and (▩) Shetland.

naturally high in cadmium and crabs injected according to the method given below.

Preparation of mammalian and fish cadmium metallothionein is generally straightforward; the method of Kägi et al. (11) invariably gives good results. Briefly, the high speed supernatant from kidney or liver is chromatographed on Sephadex G-75 in 20 mM Tris-HCl, pH 8.6. The cadmium-containing peak is applied directly to a column of DEAE-cellulose, eluted with a Tris-HCl gradient, and the product concentrated, desalted and lyophilized. With metallothionein from the crab *Cancer pagurus* this procedure failed at the stage of DEAE-cellulose chromatography, in that the cadmium failed to bind (4). This was apparently due to proteolysis, since a mild heat treatment (60°C for 10 min) enabled the purification to proceed. However, although the final product gave an amino acid analysis typical of metallothioneins, the yield was poor, the DEAE chromatogram was poorly resolved, and the product showed a surprisingly high copper content. Use of an initial batch DEAE-cellulose adsorption enabled the heat treatment to be avoided,

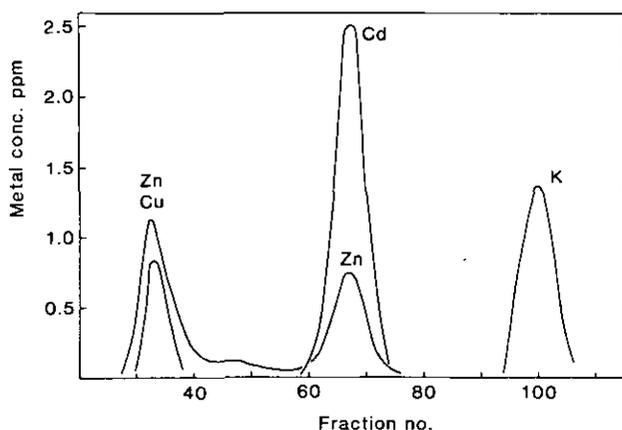


FIGURE 2. Sephadex G-75 chromatography of cytosol from the hepatopancreas of Orkney crab. Tissue metal concentrations: Cd, 28 ppm; Cu, 7 ppm; Zn, 93 ppm wet weight. Column size: 2.5 cm × 90 cm; buffer 0.01 M Tris acetate, pH 7.5; 5 mL fractions.

since the procedure rapidly removed metallothionein from the proteolytic activity (10). Subsequent purification gave a product containing a low copper content, implying that the copper present after heat treatment was an artifact due to release of copper, from either hemocyanin or other copper stores, in a form able to react with the cadmium metallothionein. The purification was carried out using Ar or N₂ gassed buffers and fractions were collected under Ar. Nevertheless, the resolution of the DEAE-cellulose chromatogram was still poor and showed three charge forms. It has recently been shown (12) that, in the presence of 0.005 M 2-mercaptoethanol, only one charge form is present in the DEAE-cellulose chromatogram of a desalted cytosol, and this is present in a well-resolved peak. This can be converted to a product which displays almost identical chromatographic properties to that found during the isolation procedure that was carried out under Ar but in the absence of mercaptoethanol. This conversion can be simply accomplished by bubbling air slowly through the same cytosol desalted in the absence of mercaptoethanol. The effect can be completely reversed by the subsequent addition to the oxidized sample of mercaptoethanol to give a concentration of 0.01 M, (Fig. 3). The simplest explanation is that use of Ar or N₂ gassed buffers and the collection of fractions under Ar was not sufficient to protect the metallothionein against a reversible oxidation. Whether this is due to inherent sensitivity of the crab metallothionein or due to oxidation enhancers (such as copper compounds) in the partially purified material has not been resolved. Preliminary experiments (Overnell, unpublished) suggest that air is capable of oxidizing purified cadmium metallothionein, although at a slower rate than in the case of the partially purified product.

This paper describes a method of purifying metallothionein that avoids the problems mentioned above, and describes some of the properties of the purified material.

Materials and Methods

Chemicals

Standard solutions of metals for atomic absorption were from British Drug Houses. Sephadex G-75 was from Pharmacia, and DEAE-cellulose was Whatman type DE-52. Ultrafiltration membranes were from Amicon Corp., type YM-2.

Crabs

Crabs were purchased locally and were maintained in circulating seawater tanks at a temperature of between 3 and 10°C and fed regularly with live mussels.

Induction of Metallothionein

Animals were injected intramuscularly with cadmium chloride in 1.1% NaCl at the base of a leg at a dose of 5 mg cadmium/kg live weight once a week for from 3 to 5 weeks, followed by at least a further 4 weeks with no injection before they were killed.

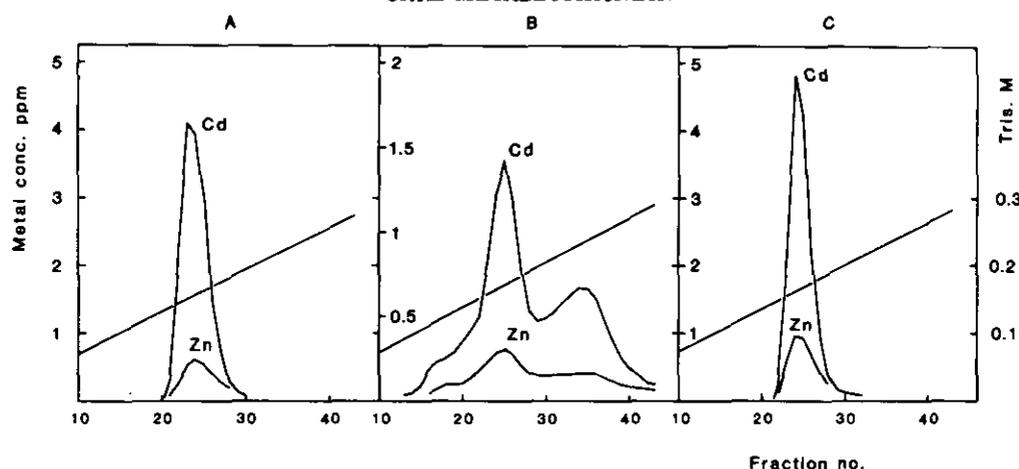


FIGURE 3. DEAE-cellulose chromatograms of desalted products from 2-mL aliquots of hepatopancreas cytosol from a cadmium-treated crab with the cytosolic concentrations Cd, 28 ppm; Zn, 13 ppm and Cu, 4.9 ppm: (A) in the presence of 0.005 M mercaptoethanol; (B) in the absence of mercaptoethanol after gentle aeration in the absence of mercaptoethanol; (C) in the presence of 0.005 M mercaptoethanol; aerated product after subsequent incubation with mercaptoethanol (0.01 M). Column dimensions: 0.9 cm \times 8 cm.

Preparation of Metallothionein

Digestive glands from cadmium-injected crabs were removed and homogenized with twice their weight of cold 0.005 M 2-mercaptoethanol. The homogenate was centrifuged at 100,000 g for 1 hr and a sample taken for metal analysis. The cytosol was diluted approximately ten times with cold 0.005 M mercaptoethanol, treated with 30 g of DEAE-cellulose for each 100 g of tissue used, and the mixture stirred intermittently for approximately 30 min. The mixture was centrifuged at 2000 rpm for 15 min and the supernatant treated with a further 30 g of DEAE-cellulose. Both extracts were packed into a column and eluted with 0.4 M Tris HCl, 0.005 M mercaptoethanol, pH 8.6. Fractions of 25 mL each were taken, and the high Cd fractions combined and applied to a G-75 column equilibrated with 0.02 M Tris-HCl, 0.005 M mercaptoethanol, pH 8.6, gassed with Ar, and eluted with the same buffer. The main Cd peak (i.e., fractions with greater than half the maximum cadmium concentration) were pooled, applied to a DEAE-cellulose column and eluted with a linear gradient from 0.02 M Tris-HCl containing 0.005 M mercaptoethanol, 20 ppm potassium, and gassed with Ar, to 0.3 M Tris-HCl containing 0.005 M mercaptoethanol, 30 ppm K, pH 8.6, and gassed with Ar. The main Cd peak was either pooled or divided into peak and tail regions and concentrated in a pressure cell using a YM-2 membrane. The concentrate was desalted on a G-75 column equilibrated with 0.01 M ammonium bicarbonate gassed with Ar, and eluted with the same buffer. The main Cd-containing fractions were pooled and lyophilized.

Amino Acid Analysis

Quantitative amino acid analysis was carried out using both a JEOL JLC 6AH (Aberdeen) and a Durrum analyzer (Zurich). Samples were oxidized before hydrolysis with performic acid (prepared from 100% formic acid to which was added 5% (v/v) 100 volume hydrogen peroxide)

overnight at 4°C. Hydrolysis was in 6 N HCl at 110°C for 20 hr or 100°C for 24 hr. After analysis, 5% and 10% corrections were applied to the values obtained for Thr and Ser, respectively.

High Pressure Liquid Chromatography

HPLC analysis was carried out using a LiChrosorb RP-18 10 μ m particle size column, 4.6 mm diameter \times 250 mm, and an LKB 215 HPLC chromatography system: buffer A: 25 mM Tris-HCl, pH 7.5; buffer B: 25 mM Tris-HCl, pH 7.5, containing 60% (w/v) acetonitrile; gradient: 0% B-25% B in 60 min. Absorbance measurements were made at 220 nm using a 1 cm path length, 10 μ L flow cell.

Results and Discussion

The preparative procedure given in the "Materials and Methods" section has enabled good yields of metallothionein to be obtained and it would appear to be the best method to date for the preparation of cadmium metallothionein from the crab *Cancer pagurus*. The method uses an initial batch absorption on to DEAE-cellulose, followed by G-75 and DEAE-cellulose chromatography. The buffers used in all these preparative steps contained 0.005 M mercaptoethanol and were gassed with nitrogen or argon. The inert gas was used to prevent the slow oxidation of the dilute mercaptoethanol by the dissolved oxygen, and the mercaptoethanol presumably maintained all the protein sulfhydryl groups in a reduced state. Figure 4 shows a typical DEAE-cellulose chromatogram. In many such cases a shoulder on the high ionic strength side of the main peak of the cadmium profile is apparent. This has not been further investigated, but seems to be also a metallothionein that probably differs from the main component by less than a single charge unit (by analogy with the published chromatograms of other mixtures of metallothioneins whose amino acid sequences have been determined). The main peak was further purified and

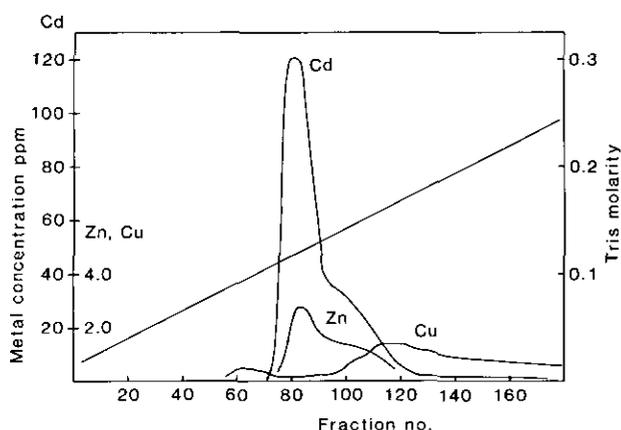


FIGURE 4. DEAE-cellulose chromatogram of pooled fractions from G-75 chromatography. Column size: 2.5 cm \times 14.5 cm. Tris gradient: from 0.02 M Tris-HCl containing 0.005 M mercaptoethanol, 20 ppm potassium, pH 8.6, and gassed with Ar, to 0.3 M Tris-HCl containing 0.005 M mercaptoethanol, 300 ppm potassium, pH 8.6, and gassed with Ar (1L + 1L). The gradient was determined from the potassium concentrations of the fractions.

desalted on a column of Sephadex G-75 equilibrated with 0.01 M ammonium bicarbonate, and then lyophilized. The final lyophilized product readily dissolved in water, but failed to do so if mercaptoethanol was present in the final buffer. The purified metallothionein showed only a single, slightly diffuse band on nondenaturing gel electrophoresis, and there was no evidence for contamination by nonmetallothionein protein.

Figure 5 is the HPLC chromatogram of purified cadmium metallothionein at neutral pH [method of Klauser et al. (13)]. It can be seen that there is only one major component (1) and a minor component (2). The other small peaks are probably artifacts since they do not change significantly with the amount of metallothionein applied. Clearly, until both these components are separated and characterized, it cannot be concluded with certainty that the minor component is indeed a metallothionein. However, the high Cys content of the amino acid analysis (see below) and the absence of Val, Ile, Leu, Phe, Tyr, and Arg does not allow for significant nonmetallothionein contamination.

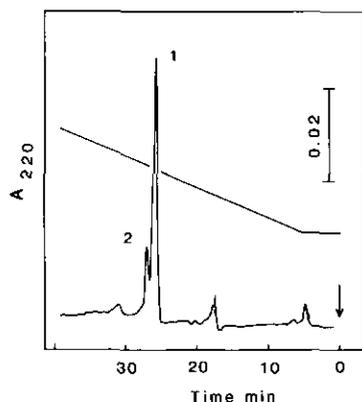


FIGURE 5. HPLC of purified crab metallothionein. The amount injected (at arrow) was 400 μ L of Tris buffer containing 10 μ L of 1 mg/mL metallothionein. Start buffer: 100% buffer A. Gradient: 0% B-25% B in 60 min. Flow rate: 1 mL/min.

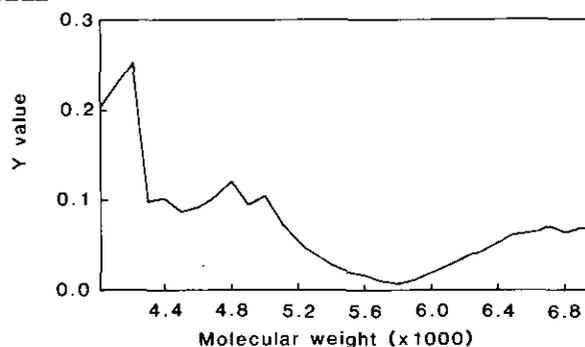


FIGURE 6. Plot of Y value as a function of molecular weight, using amino acid analysis data of purified crab metallothionein. Y value calculated by the method of Delaage (14) and molecular weight incremented in units of 100.

Amino acid analysis data were analyzed by the method of Delaage (14) to give estimates of minimum molecular weights. Six individual analyses gave minimum residuals (Y) in the range 5600 to 5800 with the unweighted mean at 5750. The lowest minimum residual was at a value of 5800, (Fig. 6). The average amino acid composition based on a molecular weight of 5800 is given in Table 1. Values are means of six determinations \pm standard deviations, followed by the nearest whole number. The result suggests that there is a total of 55 residues in the protein. Even with perfect amino acid analyses, noninteger values would be expected, since the presence of an isoprotein is strongly suggested by the HPLC results.

Metal analysis of two batches showed that cadmium was the predominant metal. By correlating metal analyses to the quantitative amino acid analysis and assuming 55 residues per molecule, it was determined that the total metal-to-protein ratio varied from 5.9 to 6.1 gram-atoms per mole of protein; zinc values ranged from 0.2 to 0.3, and copper values were very low, 0.02 to 0.03.

The ultraviolet spectrum of a sample of crab metallothionein is shown in Figure 7. At neutral pH it can be seen that two bands are probably present in the tail region of the strong 200 nm peptide absorption (spectrum b). The first derivative spectrum (inset) also shows clear evidence for two bands at approximately 235 and 252 nm, due to cadmium-sulfur charge transfer. The 235 nm band is probably related to the presence of the bridging sulfur atoms present in the clustered, fully cadmium-loaded structure, since this band is not seen in the partially cadmium-filled molecule at neutral pH nor in the holo-

Table 1. Amino acid composition of crab metallothionein.

Amino acid	Number of residues ^a	Standard deviation	Nearest whole number
Cys	18.79	0.25	19
Asp	5.18	0.07	5
Thr	4.02	0.11	4
Ser	4.84	0.33	5
Glu	2.89	0.18	3
Pro	4.34	0.25	4
Gly	4.07	0.15	4
Ala	2.01	0.07	2
Lys	9.22	0.32	9

^a Values are means of six determinations.

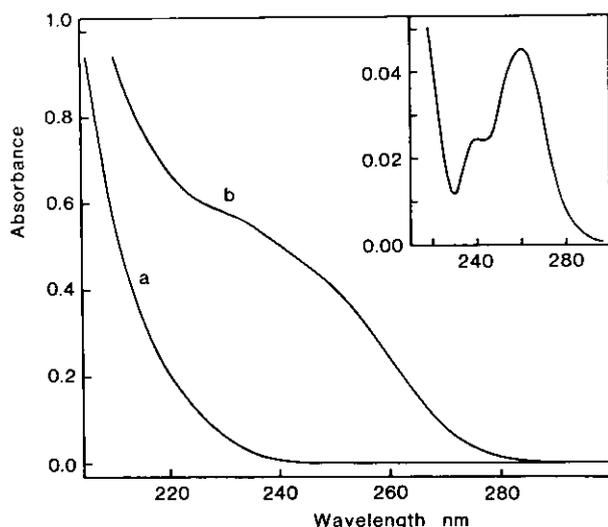


FIGURE 7. Ultraviolet absorption spectrum of 4.9 μM crab metallothionein (a) in 0.05 N HCl pH 1.5 and (b) in 0.01 M Tris-HCl, pH 8.4. Inset: first derivative spectrum of (b). 1 cm path length.

protein at lower pH when some metal is lost (Overnell and Vařák, unpublished; Overnell, unpublished). At acid pH these bands are absent due to displacement of the metal atoms by protons (spectrum a). Quantitative determination of the absorbance at 220 nm and 250 nm combined with the amino acid analysis results yields the following extinction coefficients: pH 1.5, $\epsilon_{220} = 50,000$ and pH 8.4, $\epsilon_{250} = 92,000$.

Conclusion

Two modifications to the commonly used isolation procedure for vertebrate metallothionein are required for the isolation of crab metallothionein in good yield. The first is the use of an initial rapid DEAE-cellulose batch absorption-desorption step to remove metallothionein from digestive proteases found in the cytosol. The second is the use of 5 mM 2-mercaptoethanol in all buffers except that used for the final desalting chromatography. All buffers should be gassed with argon or nitrogen. The product appears to contain only one band on nondenaturing polyacrylamide gel electrophoresis, and two components, one major one minor, as judged by HPLC.

These results may be compared with those obtained for the metallothioneins from the crab *Scylla serrata*. The two isoproteins were shown by sequence determination to contain 58 and 57 amino acids (15) and 18 cysteine residues. A value of 6 was obtained for the number of metal atoms per molecule (16). The amino acid analysis data from *Cancer pagurus* would suggest a value of 55 for the total number of amino acids, a value of 19 for the

number of cysteine groups, and a total metal-to-protein ratio of 6 gram-atoms per mole.

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