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Cadmium–Binding Proteins in Midgut Gland of Freshwater Crayfish *Procambarus clarkii*

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Metallothioneins, metal binding proteins, were originally isolated and characterized by Margoshes and Vallee (1975). This proteins have a high affinity for various heavy metals, particulary cadmium and mercury (Roesijadi 1980) and have extensively been studied in mammals (Kägi and Nördberg 1979). Metal binding proteins have been observed in a variety of marine invertebrates (Roesijadi 1980); however, there is very little information available on metal binding proteins in freshwater invertebrates, and particulary in freshwater crustaceans (Lyon et al. 1983; Lyon 1984).

The presence of such proteins has been variosly suggested as indicating involvement in uptake, storage, transport, and elimination of toxic metals (Engel and Brower 1982) and in the routine metabolism of metals (Roesijadi 1980)

Cadmium is a ubiquitous non essential element which possesses high toxicity to aquatic organisms (Lake et al. 1979; Lalande and Pinel-Alloul 1984). Cadmium binding proteins observed in invertebrates have similar characteristics to mammalian metallothioneins: low molecular weight (10,000-12,000), high cysteine content, stable to acid and heat treatment, inducible by metal exposure, low ultraviolet absorption at 280 nm (contain few aromatic amino acids) and high absorption at 254 nm, a characteristic absorbance of the mercaptide-metal bond. This characteristic absorbance disappeared on acidification and reappeared with neutralization (Kägi and Nordberg 1979; Frazier et al. 1985).

In 1978, the American red crayfish appeared in Albufera Lake and the surrounding rice fields (Valencia, Spain). Albufera Lake and the surrounding rice fields waters are subjected to very heavy loads of sewage and toxic industrial residues (including heavy metals) from the many urban and wastewaters in this area.

In previous reports we studied the toxicity and accumulation of cadmium on Procambarus clarkii of Albufera Lake. This crayfish shows a high resistence to cadmium and a great accumulation rate of this metal in several tissues, including midgut gland (Del Ramo et al. 1987; Díaz-Mayans 1986). The midgut gland is a major site for the accumulation of metals, and it has been suggested that this tissue may be the site of metal detoxification system (Lyon et al. 1983). Since Procambarus clarkii shows a high resistence to cadmium, the presence of cadmium binding proteins (Cd-BP) in midgut gland of these crayfish would be expected.

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This report describes results on the characterization of Cd-BPs obtained from cadmium exposed crayfish Procambarus clarkii, demostrating their presence in this freshwater crayfish.

MATERIALS AND METHODS

Adult intermolt specimens of the crayfish Procambarus clarkii (Girard) were collected in Albufera Lake and transported immediately to the laboratory where they were transferred to 300-L aquaria for 15 days and maintained before treatment at 23°C with daily diet of pork liver.

A variety of techniques have been applied to the study of metal binding protein properties. In this study we used, gel filtration chromatography, which produces good separation of the fractions and permits the easy identification of metal binding proteins. Samples were eluted in a series corresponding to their molecular weights.

Many studies on metal binding proteins have been performed using Sephadex G-75. The resolving properties of this particular gel type, however, are not totally suitable for separating Cd-BPs from other low molecular weight impurities for cadmium binding proteins elute near the lower limit (5000 mol wt) of the linear fractionating range. Many elutions have been performed under oxidizing conditions (Minkel et al 1980) causing artificial changes to elution profiles. In accordance with Wong and Rainbow (1986), we used DTT to maintain reducing conditions and PMSF to prevent protease activity and we used a gel type with a more suitable linear fractionating range such as Sephadex G-50 (30000-1500 mol wt).

Tris (hydroxymethyl) aminoethane, and cadmium solutions were purchased from E. Merck (West Germany). Dextran blue, aprotinin, cytochrome c and chymotrypsinogen protein standards, phenylmethylsulphonyl (PMSF) to prevent protease activity and dithiothreitol (DTT) to maintain reducing conditions were purchased from Sigma Chemical Company (West Germany). Sephadex G-50 resin was obtained from Pharmacia Fine Chemicals Company (Uppsala, Sweden).

Crayfish were starved for 24 h before use to minimize the effects of the digestive cycle. Induction of Cd-BP was achieved by injection at the base of the leg with cadmium chloride at a dose of 2.5 mg of cadmium per kg once a week for 2 wk, followed by a least 1 wk with no injection, before killing.

The midgut gland of experimental animals was dissected out of the body, blotted and weighed. In accordance with followed method by Engel and Brouwer (1984) (with minor changes) two midgut glands were minced and homogenized in a approximately equal volume of homogenizing buffer, Tris-HCl, (0.06 M Tris, 0.01 M NaCl, HCl added to adjust the pH to 8.6) with 0.1 mM PMSF to prevent protease activity and 1 mM DTT to maintain reducing conditions.

The homogenate was centrifuged at 30,000 g for 45 min at 4°C. The supernatant was heat treated at 60°C for 10 min and centrifuged again at 30,000 g for 45 min at 4°C. The resulting supernatant was filtered with a 0.22 µm cellulose filter (Millipore) to remove lipid material and stored at -80°C prior to gel permeation chromatography.

A 2-mL aliquot of the supernatant was then applied to a column of sephadex G-50 (2.6 x 60 cm) preequilibrated (at 4°C) with 0.06 M Tris, 0.01 M NaCl, pH 8.6. The column was eluted with the same buffer and 0.5 mM DTT at a constant rate of

50 mL/h during which time 4 mL fractions were collected. UV absorbance was measured on a Bausch & Lomb Spectronic 2000. Absorbances were measured at 254 and 280 nm and 254/280 ratios were established. Spectral changes on acidification and neutralization proteins fractions were determined.

Cadmium concentrations were determined by flame in a Perkin-Elmer model 5000 atomic absorption spectrophotometer equipped with a model 561 recorder, a deuterium background corrector. In control animals where was determined by graphite furnace.

RESULTS AND DISCUSSION

Figure 1 is a Sephadex G-50 elution profile derived from midgut gland tissue of control animals. No significant levels of cadmium occur in this chromatographic separation.

Figure 2 is a typical Sephadex G-50 (linear separation range 30,000-1,500 mol wt) elution profile derived from midgut gland tissue pooled from two female crayfish. No significant levels of cadmium occur in the void volume. One cadmium peak is clearly resolved. Cadmium was accumulated in the low molecular weight fraction corresponding to the molecular weight range of 10,000 to 12000. This fraction had high ultraviolet absorption at 254 nm and a higher 254/280 ratio. The 254 nm absorbance reflects the presence of Cd-BP as a result of the characteristic absorbance of the mercaptide-metal bond (Kägi & Nördberg 1979).

Cd-BP fraction were scanned on a U.V. absorbance spectrophotometer and a maximal absorbance at about 254 nm with minimal absorbance at 280 nm were found indicating a lack of aromatic amino acids. This maximal absorbance disappeared on acidification and reappeared with neutralization (Figure 3) indicating the presence of a mercaptide-metal bond. These results show the presence of a Cd-BP in midgut gland of freshwater crayfish Procambarus clarkii treated with cadmium.

In previous report (Díaz-Mayans et al. 1986) using crayfish collected from Albufera Lake of Valencia, which were maintained in similar conditions as the control crayfish used in the present study, we found that the midgut gland showed total amounts of cadmium about 0.5 µg/g dry weight. In control animals (see Figure 1) there was not found significant levels of cadmium in the low molecular weight fractions. Furthermore, protein peaks in this elution profile are not resolved. These results suggest that low levels of cadmium, as well as 0.5 µg/g dry weight, are not able to induce the Cd-BP protein synthesis.

Midgut gland samples of crayfish injected with high amounts of cadmium as cadmium chloride showed about 100 µg/g dry weight of cadmium. This level of cadmium content in tissue induced cadmium binding proteins. It can be clearly detected by our method (see Figure 2).

Marine crustaceans appear to possess metal binding proteins (Overnell and Trewhella 1979; Engel and Brouwer 1984; Wong and Rainbow 1986) while in freshwater crustaceans there are not evidence that the presence of these proteins.

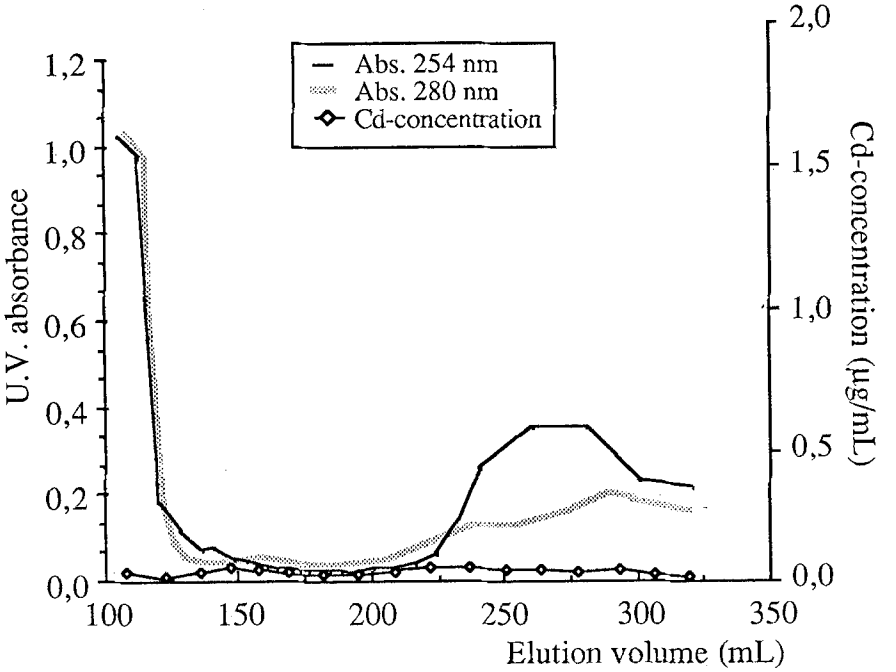

Figure 1. Sephadex G-50 elution profile for a midgut gland of control animal. Sample was homogenized in Tris-HCl buffer with 0.1 mM PSMF and 1 mM DTT, and eluted in Tris-HCl buffer with 1 mM DTT added

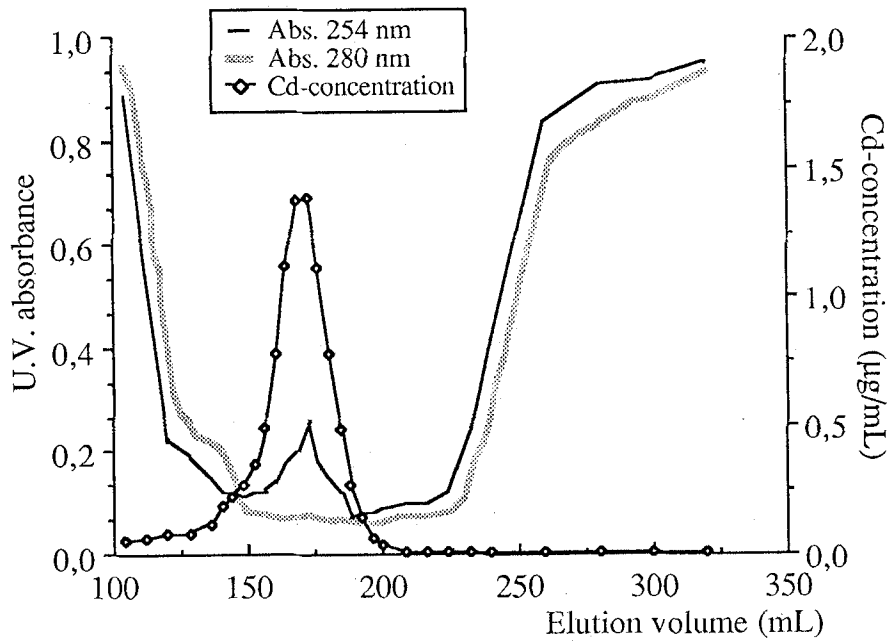

Figure 2. Sephadex G-50 elution profile for a midgut gland of terated animal. Sample was homogenized in Tris-HCl buffer with 0.1 mM PSMF and 1 mM DTT, and eluted in Tris-HCl buffer with 1 mM DTT added

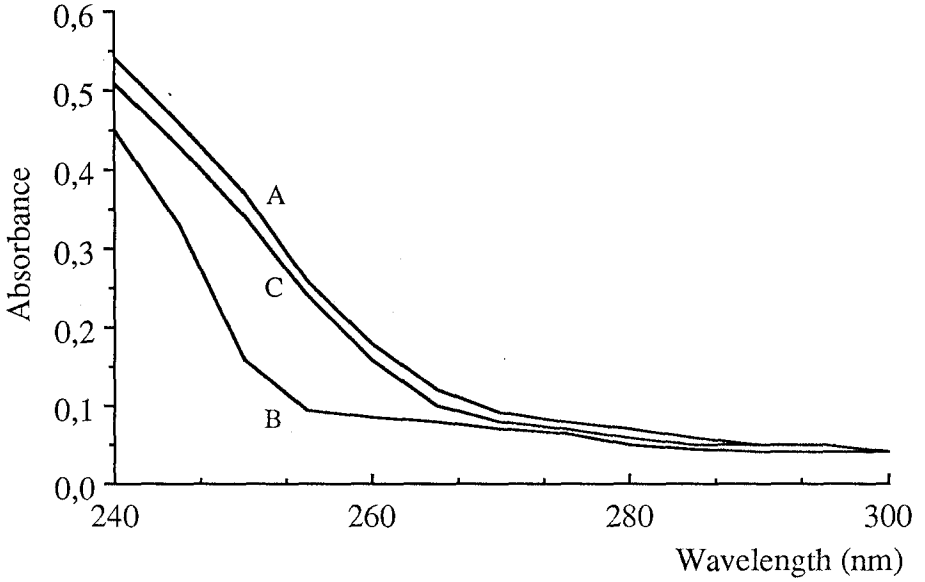

Figure 3. UV Absorbance spectra of cadmium binding proteins peak subjected to acidification and neutralization. A: pH=8.5; B: after acidification to pH=2; C: after addition of base to pH=8.5.

Lyon et al. (1983) have been reported that there are not typical metallothioneins induced by cadmium in the crayfish Austropotamobius pallipes. On the contrary, in midgut gland of Procambarus clarkii, we have found the presence of binding proteins (low molecular weight 10,000-12,000) which possess characteristic ultraviolet absorbance at 254 nm.

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