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Chromatographic analysis of low-molecular-mass copper-binding ligands from the crab species *Scylla serrata* and *Portunus pelagicus*

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ABSTRACT

Two copper-binding proteins and a zinc-binding ligand were isolated from the hepatopancreas of the crab *Portunus pelagicus*. The copper-binding proteins behave similarly to those from the crabs *Carcinus maenas* and *Scylla serrata*, and were shown to be metallothioneins. Reversed-phase high-performance liquid chromatographic (HPLC) analysis confirmed the relative purity of both proteins with only cross-contamination between the two different forms of metallothioneins, and offers a good method to separate the two forms of metallothioneins. The vast difference in the retention times (and hence the hydrophobicity) in reversed-phase HPLC indicates that the two proteins could be conformationally very different.

INTRODUCTION

Copper and zinc are essential in trace amounts for all life forms, but are toxic when present at inappropriately high concentrations. Metallothioneins are a group of low-molecular-mass, cysteine-rich metal-binding proteins which are thought to occupy a central position in the metabolism of particular trace metals such as copper, cadmium, zinc and mercury [1]. They were first isolated from mammalian species [2] but have since been found in other organisms.

Metallothioneins have also been found in crab species. Two forms of metallothionein proteins (molecular mass 10 000 and 4100) have been isolated from the hepatopancreas of the shore crab *Carcinus maenas* [3], showing great individual variability between crabs as to their presence and their contents of copper, zinc and cadmium. The complete amino acid sequences of metallothioneins 1 and 2 from the crab *Scylla serrata* have also been reported [4]. Comparison of their primary structures with the known sequences of thioneins from equine kidney [5], human liver [6] and mouse liver [7] reveals a high degree of sequence homology among the six proteins, especially with respect to the conservation of the abundant cysteinyl residues.

Copper is regulated in trace levels in most vertebrates and is associated with very specific enzymes. However, numerous invertebrate species, including crustaceans, rely on haemocyanin, a Cu^{2+}-containing molecule [8]. Thus, in these species, Cu^{2+} is incorporated in metabolic and physiological activities in substantial amounts.

As metallothioneins are induced by and bind copper, they could play a central role in copper metabolism in most organisms. However, metallothioneins may be only part of a complex interplay of different compartments for trace metals, as they are ubiquitious in not being able to discriminate between copper, zinc and cadmium.

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EXPERIMENTAL

In the laboratory, live male crabs obtained from local markets were maintained in separate compartments in plastic tanks in aerated artificial sea water (Marinemix), prepared at a salinity of 33.3% and containing 0.5 μg/l of Cu^{2+}, for 2-week periods. The metal was added to the medium as an aliquot of a stock solution of the analytical-reagent grade salt. After the 2-week exposure period, the crabs were killed by freezing and the hepatopancreas was dissected out from each on partical thawing.

It was necessary to maintain reducing conditions throughout extraction with dithiothreitol (DTT) [4], as metallothionein proteins are very prone to the effects of oxidation during isolation owing to their very high cysteine content. To the dissected tissue was added an approximately equal volume of homogenizing buffer, Tris–HCl (0.01 *M* Tris, 0.01 *M* NaCl, HCl added to adjust the pH to 8.6) with 0.1 m*M* phenylmethanesulphonyl fluoride (PMSF) to prevent protease activity and 1 m*M* DTT to maintain reducing conditions. The mixture was homogenized and then centrifuged at 25 000 *g* for 3 h.

The supernatant was applied to a Sephadex G-50 column and eluted with Tris–HCl buffer (pH 8.6) containing 0.5 m*M* DTT to maintain reducing conditions. Pooled fractions corresponding to peaks I and II were then separately loaded on to an ion-exchange column (DEAE-Sephacel) and eluted with a 0.02 *M* Tris–HCl buffer gradient of increasing ionic strength (conductivity 2–35 mS, 0.01–0.4 *M* NaCl). The Cu^{2+}-containing fractions were then freeze-dried and desalted by passage through a Sephadex G-25 column. Metal analysis by flame and graphite furnace atomic absorption spectrophotometry (AAS) with a Shimadzu AA 670 instrument was performed after each purification step to check for the elution position of the metal-binding ligands.

The desalted fractions were then analysed by high-performance liquid chromatography (HPLC) using a Waters reversed-phase C_{18} (Nova-Pak C_{18}, 4 μm, 100 Å) column (300 × 3.9 mm I.D.) with a binary gradient Shimadzu HPLC LC-6A system. Buffer A was 0.1% trifluoroacetic acid (TFA) in water purified with a Milli-Q system (Millipore) and buffer B was 0.1% TFA in HPLC-grade acetonitrile.

Capacity factors, k', were calculated from the equation $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the elution times of a retained and non-retained sample, respectively.

RESULTS AND DISCUSSION

Edible crabs are heavily consumed in the coastal areas in Asia. They are of special interest as the accumulation of heavy metals within their body tissues as a result of high concentrations of metals in sea water from polluting effluents could result in levels unacceptable for human consumption. There is therefore a need to monitor total levels, and also to study further the mechanisms of metal regulation in these tropical decapods.

Although much work has been done on temperate species of crabs, work on tropical species is conspicuous by its absence. This paper serves to redress this imbalance by identifying and isolating trace-metal-binding, low-molecular-mass ligands in selected tropical species of organisms, with the ultimate aim of understanding the proper role of these ligands in the complex physiology of these organisms. We decided to use a local crab species, *Portunus pelagicus*, owing to their ready availability, the large size of the hepatopancreas, convenience and the fact that these ligands have been shown to occur in crabs. In addition, we also obtained a few *Scylla serrata* crabs for comparison studies.

The *Scylla serrata* species of crabs have been studied previously and the metallothioneins from this species have been characterized and sequenced [4]. The chromatographic behaviour of the material obtained from *Portunus pelagicus* is very similar to that from *Scylla serrata* and *Carcinus maenas* crabs [3].

Fig. 1 shows a typical Sephadex G-50 elution profile (molecular mass linear separation range 30 000–1500) derived from hepatopancreas tissue pooled from male *Portunus pelagicus* crabs, reducing conditions being maintained throughout by the addition of DTT. Peaks I and II correspond to metallothioneins as seen in the *Scylla serrata* and *Carcinus maenas* crabs. Although the two peaks are not completely resolved, there is a definite shoulder indicating the presence of two proteins. Peak III binds to zinc, corresponding to a low-molecular-mass non-proteinaceous zinc-binding ligand that had also been found in the latter two species of crabs [9].

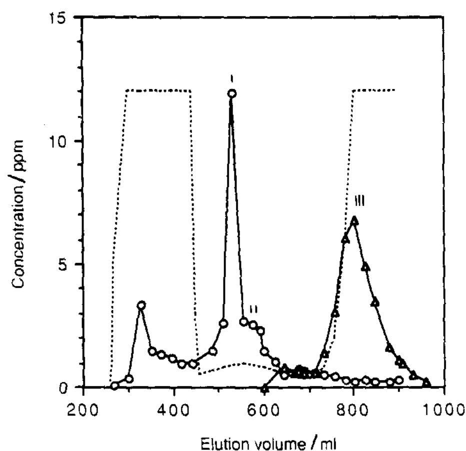

Fig. 1. Sephadex G-50 elution profile of male *Portunus pelagicus* crab hepatopancreas from copper-exposure experiments. The sample was homogenized in Tris–HCl buffer containing 1 m*M* DTT and eluted with Tris–HCl buffer containing 0.5 m*M* DTT. Bed volume, 780 ml; flow-rate, 70 ml/h; detection wavelength, 254 nm. ○ = Copper; △ = zinc. Dashed line, absorbance.

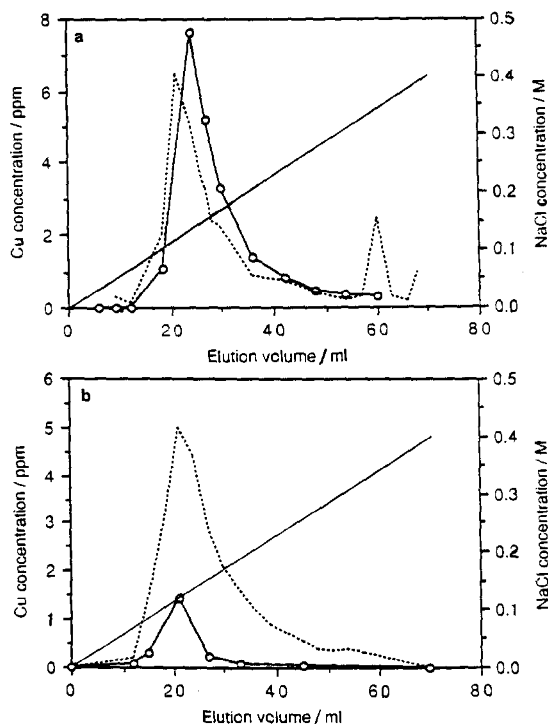

Fig. 2. Elution profile of Sephadex G-50 fractions of (a) peak I and (b) peak II on a DEAE-Sephacel ion-exchange column (12 × 1.2 cm I.D.). Bed volume, 12.5 ml; flow-rate, 18 ml/h; detection wavelength, 254 nm. ○ = Copper. Dashed line, absorbance.

Fig. 2 shows typical ion-exchange chromatograms of material obtained from peaks I and II of the Sephadex G-50 elution and applied separately to a DEAE-Sephacel ion-exchange column (12 × 1.2 cm I.D.). Olafson *et al.* [10] reported two well resolved metal-containing peaks using a 30 × 2.6 cm I.D. Bio-Rad Cellex D DEAE-cellulose column using pooled fractions isolated by gel permeation chromatography. As peaks I and II were not completely resolved peaks on the Sephadex G-50 column, we expected material from peaks I and II to be mixtures of the two metallothioneins. However, we observed that on our DEAE-Sephacel column, the material from both peaks I and II gave single metal-containing peaks on anion exchange. That the two metallothioneins were not resolved at this stage in the purification could be due to the sharp gradient employed on the short ion-exchange column used in the elution of the proteins. Further studies are currently being conducted to examine this more closely. The proteins were desalted with water through a Sephadex G-25 column, with salt detected by conductivity measurements and the metallothionein proteins by AAS.

Analytical reversed-phase HPLC was carried out on purified material (from the *Portunus pelagicus* crabs) from peaks I and II separately to check for homogeneity. For comparison, the material from the *Scylla serrata* crabs was also subjected to the same analysis. Fig. 3a and b show the respective chromatograms for the two species of crabs. The capacity factors (k') of the two proteins from the *Portnunus pelagicus* crabs (2.5 and 8.4) are very similar to those for the proteins isolated from the *Scylla serrata* crabs (2.7 and 8.3), indicating that the two proteins from the hepatopancreas of the *Portunus pelagicus* crabs are therefore metallothionein proteins.

As peaks I and II were not completely resolved on the Sephadex G-50 column, it is not surprising that in both instances the material from the two peaks appeared to contain a mixture of the same two proteins. From the chromatograms, it is obvious that although both peaks consist of mixtures of the two proteins, peak II consists predominantly the protein of $k' = 2.5$. As can be seen from the metal trace using AAS measurement in Fig. 1, peak I is a sharp peak whereas peak II is relatively broad, and it appears that elution of peak II has begun before

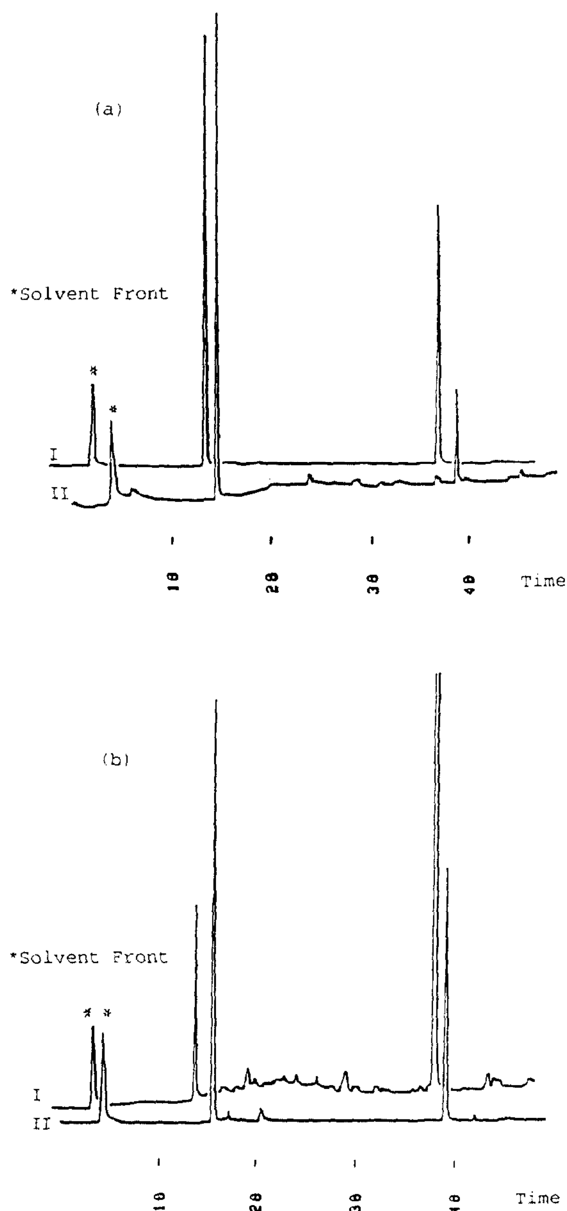

Fig. 3. HPLC analyses of metallothioneins from peaks I and II of (a) *Portunus pelagicus* and (b) *Scylla serrata* on a reversed-phase C_{18} column. Solvents used in elution: (A) 0.1% TFA in Milli-Q-purified water and (B) 0.1% TFA in acetonitrile, with a gradient from 10 to 60% B in 40 min. Flow-rate, 0.5 ml/min; detection wavelength, 254 nm. Time in min.

peak I is completely eluted. Therefore, it is understandable that there is cross-contamination and that peak II is purer than peak I. From this analysis, it is obvious that reversed-phase HPLC would be a good method to isolate pure samples of the two proteins because of the vast difference in the retentions of the two proteins on the reversed-phase C_{18} column used.

In addition to these two metallothionein proteins, hepatopancreas extracts typically contain a zinc-binding complex of low molecular mass, as has also been shown with *Carcinus maenas* [9]. The characterization of this zinc-binding ligand will be presented in a separate paper. We have also observed that the level of induction of the proteins after exposure for 2–5 days to artificial sea water spiked with Cu^{2+} appears to be higher than that for a longer period (14 days). This is based on the results of measurement of metal levels after the first purification step (Sephadex G-50). We are now carrying out time-scale experiments to verify this observation.

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