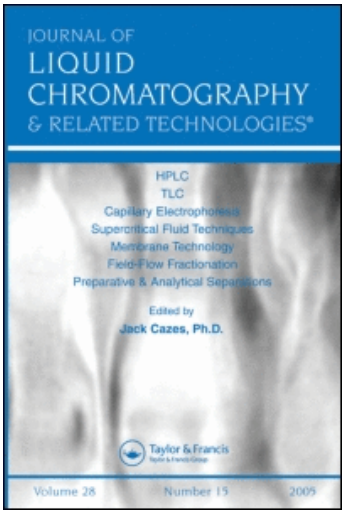

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Siau-Gek Ang[a]; Victor W. T. Wong[b]
[a] Department of Chemistry, National University of Singapore, Kent Ridge, Singapore [b] Department of Zoology, National University of Singapore, Kent Ridge, Singapore

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CHROMATOGRAPHIC ANALYSIS OF METALLOTHIONEIN PROTEINS FROM THE CRAB SPECIES *SCYLLA SERRATA* AND *PORTUNUS PELAGICUS*

SIAU-GEK ANG[1]* AND VICTOR W. T. WONG[2]
[1]*Department of Chemistry*
[2]*Department of Zoology*
National University of Singapore
Kent Ridge
Singapore 0511

ABSTRACT

Different chromatographic techniques have been used successfully to isolate and purify two copper-binding proteins and a zinc-binding ligand from the hepatopancreas of the crab *Portunus pelagicus*. Comparison studies were made using the crab *Scylla serrata* which had been studied previously. Based on the identical purification protocol and the very similar retention times on reversed phase HPLC, the copper-binding proteins are found to be similar to those from the crab *Scylla serrata* and are shown to be metallothioneins. Reversed phase HPLC analysis confirms that there are two distinct forms as purified by standard molecular sieve and ion exchange chromatography.

* Author to whom correspondence should be addressed.

INTRODUCTION

Rapid size exclusion chromatography (SCE) employing siliceous supports as stationary phases and aqueous buffers as mobile phases has often been used for analytical and preparative separations of proteins, owing to its simplicity. Gel filtration and ion exchange have been used in the purification of the metallothionein proteins successfully (1,2).

The metallothioneins (MTs) are a group of cysteine-rich metal-binding proteins (3) and they are thought to occupy a central position in the metabolism of essential trace metals. The functional aspects of these ligands are not clearly understood although a role as a detoxification mechanism for trace metals has not been ruled out. Whilst it is clear that their discriminatory properties may allow them to bind to selected groups of closely-related trace metals, they are unable to discriminate between the different trace metals within these groups. Since trace metals must be closely regulated, it would be interesting to see how this is brought about and where the MTs and other low molecular weight (mw) metal-binding ligands fit into the scheme.

There is some controversy over the number of such ligands in some organisms studied. Two isoforms

have been reported in the crab species, *Scylla serrata* and *Cancer magister* (1) and in the species, *Cancer pagurus* (4). More recently, Overnell (5-8) has reported resolution of metallothioneins into three isoforms in *Cancer pagurus*. The problem lies in that because of their similarity in size, it is usually not possible to use SCE to resolve the different isoforms. Although most other authors report on the isolation of several isoforms of MTs, Wong and Rainbow (2) have showed that there is the presence of at least a third low mw Zn-binding ligand that could be mistaken for MTs based on gel chromatography. We suspect that many reports on a specific Zn MT is this novel Zn-binding ligand which, after careful purification, has been shown not to be proteinaceous (9). We report here the confirmation of two distinct forms of MTs as well as the Zn-binding ligand from *Scylla serrata* and *Portunus pelagicus*.

MATERIALS

Live male crabs (*P. pelagicus* and *S. serrata species*) obtained from local markets were kept in separate compartments in plastic tanks in artificially made sea water (Marinemix) containing 0.5 ug/ml of Cu for periods of two weeks. Crabs were killed by

freezing and the hepatopancreas were dissected out on thawing.

As will be explained later, it was necessary to maintain reducing conditions throughout the isolation and purification procedure. Dithiothreitol (DTT), (2, 10, 11) was shown to be superior to 2-mercaptoethanol in terms of maintaining reducing conditions (12).

The dissected hepatopancreas is mixed with an approximately equal volume of homogenising buffer (Tris-HCl:0.01 M Tris, 0.01 M NaCl, HCl added to adjust the pH to 8.6 with 0.1 mM phenylmethylsulphonyl fluoride (PMSF) to prevent protease activity and 1 mM DTT to maintain reducing conditions). The mixture was homogenised before centrifuging at 25,000 g for 3 hours. The supernatant was applied onto a Sephadex G-50 column and eluted with Tris-HCl buffer (pH 8.6) with 0.5 mM DTT to maintain reducing conditions. Selected fractions were then loaded onto an ion-exchange column (DEAE Sephacel) eluting with a 0.02 M Tris-HCl buffer gradient of increasing ionic strength (c. 2-35 mS, 0.01-0.4M NaCl). From the ion-exchange column, selected fractions were then freeze-dried and desalted on a G-25 column with water.

Metal analysis (using flame and graphite furnace atomic absorption spectrophotometry (AAS)) of select-

ed eluted fractions was performed after each purification step to check for the eluting position of the metal-binding ligands. The fractions after desalting were analysed by reversed phase High Performance Liquid Chromatography using a Waters C18 column on a binary gradient Shimadzu HPLC system. The buffer system employed is :

Buffer A : 0.1% trifluoroacetic acid in Millipore water

Buffer B : 0.1% trifluoroacetic acid in HPLC grade acetonitrile

Gradient : 10 to 60% of B in 40 min.

RESULTS

Figure 1 shows a typical G-50 (linear separation range 30,000-1,500 mol wt) elution profile derived from hepatopancreas tissue pooled from male *Portunus pelagicus* crabs, reducing conditions being maintained throughout by the addition of DTT (10, 12). From analysis by atomic absorption spectrophotometry, two Cu-binding peaks (Peaks I and II) and one Zn-binding peak were detected. Similar observations have been made in the case of the shore crab, *Carcinus maenas* (8), where two Cu-binding MT proteins have been reported.

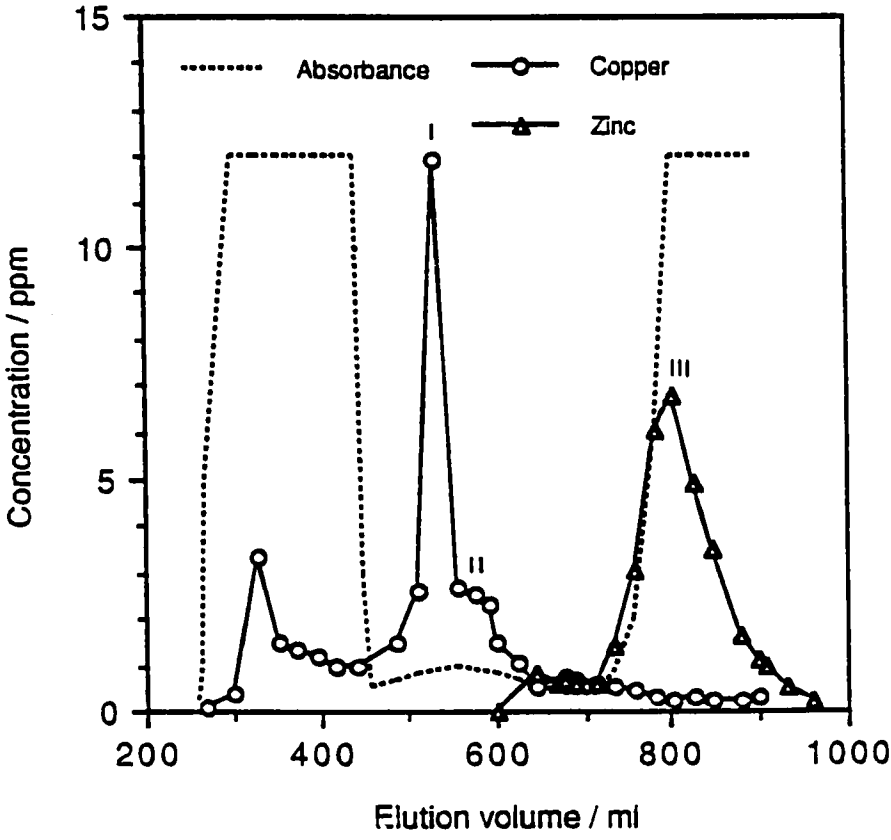


Figure 1 Sephadex G-50 elution profile of male *Portunus pelagicus* crab hepatopancreas from Cu-exposure experiments. Sample was homogenised in Tris-HCl buffer with 1 mM DTT added and eluted in Tris-HCl buffer with 0.5 mM DTT added. Bed volume = 780 ml, flow rate = 70 ml/h, detection wavelength = 254 nm.

Although the two MT-like peaks are not completely resolved in gel filtration with Sephadex G-50, pooled fractions of Peaks I and II from the Sepadex G-50 column were applied separately to an ion-ex-

change column containing DEAE Sephacel (Figure 2). The proteins were desalted with water through a Sephadex G-25 column. The salt peak was detected with conductivity measurements and the MT peak by AAS.

The purified desalted products from Peaks I and II were also subjected to analytical reversed phase HPLC to check for homogeneity (Figure 3 (a)). Figure 3(a) shows that Peak I consists of a mixture of two proteins, with retention times of 13.5 and 38.4 minutes respectively. Analysis of Peak II in the same manner shows mainly one protein (the earlier eluting protein) with a trace of the other protein. It is obvious that reversed phase semipreparative or preparative scale HPLC would offer a good alternative method to isolate pure samples of the two proteins because of the vast difference in the retention of the two proteins on the reversed phase C18 column used.

HPLC studies carried out to compare the two copper-binding proteins from *P. pelagicus* with the MTs from *S. serrata* crabs shows that the retention times of the corresponding proteins are almost identical (Figure 3(b)), confirming that the two proteins

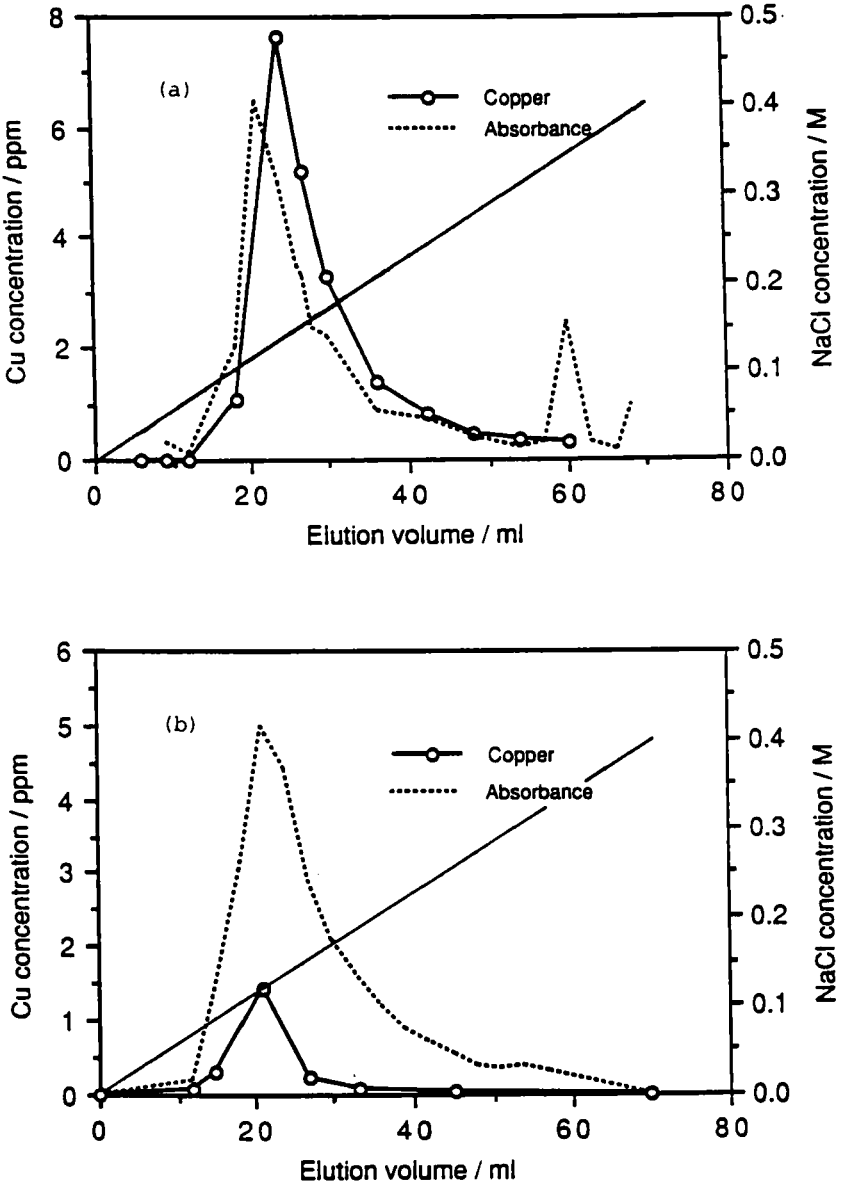


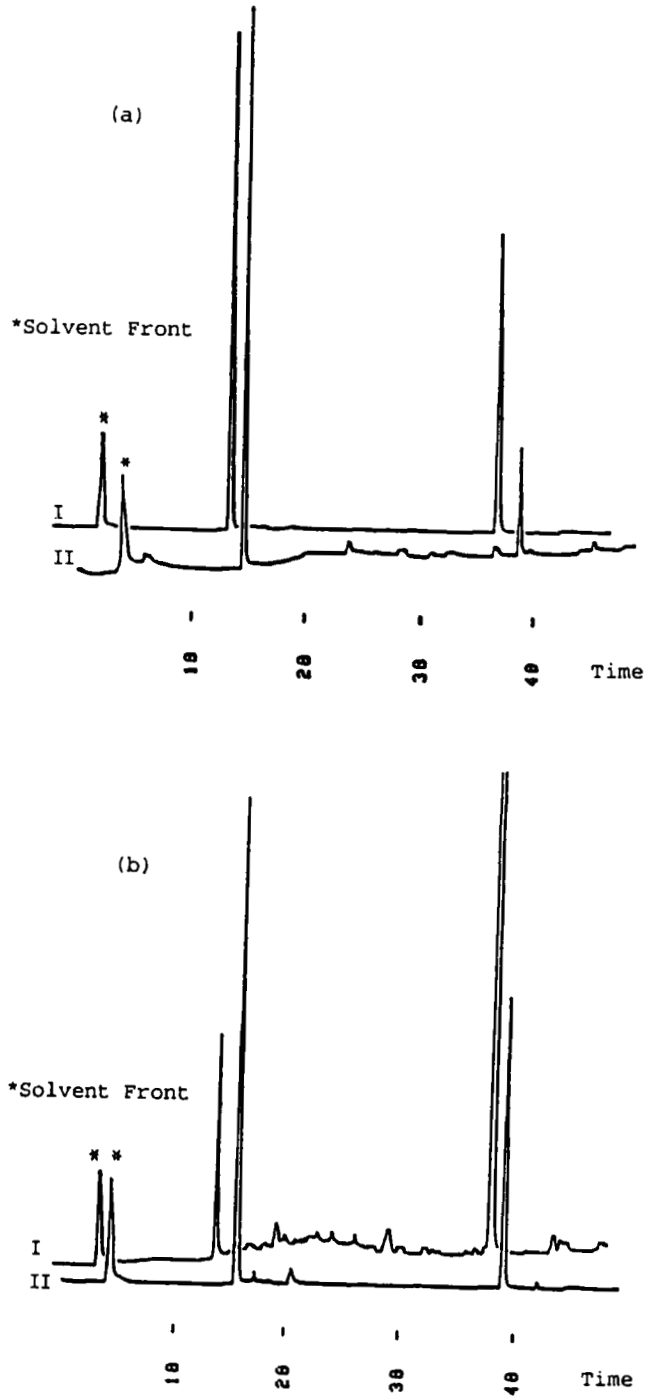
Figure 2 Elution profile of selected Sephadex G-50 fractions of (a) Peak I and (b) Peak II on a DEAE Sephacel ion-exchange column (1 x 20 cm). Bed volume = 12.5 ml, flow rate = 18 ml/h, detection wavelength = 254 nm.

from the hepatopancreas of *P. pelagicus* are therefore MT proteins.

DISCUSSION

In this work, Cu has been used to induce MTs in *P. pelagicus* because it has been shown that the relative binding affinities for metals to rat kidney metallothionein lie in the order Hg > Cu > Cd > Zn (13-15). The two-week exposure period selected was due to data (16) indicating elevated levels of MT upon metal exposure over a two week period. The novel Zn-binding ligand as found in *C. maenas* (9) and tentatively identified as a tricarboxylic metabolite (18-21) is also found in *P. pelagicus*. Its proper characterisation will be presented in a separate paper.

Metallothioneins are very prone to the effects of oxidation during isolation due to their very high cysteine content. In oxidation, disulphide bridges are formed and the MTs either copolymerize or combine with other proteins to move into the high mw fractions on Sephadex G-75 profiles (21). Such oxidation artefacts could account for the confusion in earlier work as to the number of isoforms present in a species. Therefore precautions were taken in the course of this work to maintain the ligands in a reducing environment.



The clarified supernatant of the material after centrifuging was first purified substituting Sephadex G-50 for Sephadex G-75 as it gives better resolution for low mw moieties. The G-50 profile for *Portunus pelagicus* indicates that there are two metallothionein forms that are however not completely resolved. Separation (albeit incompletely) of the forms in Sephadex G-50 indicates an apparent difference in mol. wt. for the crab MTs. We have grouped them into two classes, MT I and MT II. This is based solely upon chromatographic behaviour and does not necessarily imply structural or functional homology, especially when comparing proteins from divergent species.

For the two MTs in the species *Scylla serrata*, Lerch et al. (22) has shown that the total number of amino acid residues differ by one. Thus the apparent difference in mol. wt. may be due to either dimer formation, differences in metal saturation level resulting in conformational differences, or both. Lerch et al. (19) has shown that crab MTs can bind

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Figure 3 HPLC analyses of metallothioneins from (a) *Portunus pelagicus* and (b) *Scylla serrata* on reversed phase C18 column. Solvents used in the elution were A : 0.1% TFA in Millipore water and B : 0.1% TFA in acetonitrile. gradient : 10-60% B in 40 min, flow rate :0.5 ml/min, detection wavelength : 254 nm.

only 6 g-atoms of metal versus 7 g-atoms for vertebrate MTs. In contrast to vertebrate MTs, Roesijadi (23) has indicated that invertebrate MTs are unsaturated with metal and this was confirmed by Wong and Rainbow (11). For *Portunus pelagicus*, the G-50 profile shows a sharp peak for MTI and a broad shoulder peak for MTII. MT I may represent saturated MTs, possibly even existing as dimers, and MT II may represent a mixture of partially saturated MTs with varying conformations resulting in a sharp peak for MT I and a broad peak for MT II on gel chromatography.

Reversed phase chromatography of proteins involves separation using chemically bonded hydrophobic phases. This method is based on hydrophobic interactions between hydrocarbon chains on the stationary phase in the column and hydrophobic domains of chromatographed protein molecules in the presence of a polar mobile phase. Gradual elution of individual components of a mixture can be achieved by decreasing the polarity of the mobile phase by the addition of alcohol or acetonitrile, since hydrophobicity of peptides or proteins primarily dictates their retention in reversed phase columns.

Reversed phase HPLC was carried out in acidic conditions (ca pH 2), using 0.1% trifluoroacetic acid. In both cases, two well-separated peaks were obtained. Although the metal can be removed by exposure to low pH, graphite furnace AAS analysis confirmed that the two peaks obtained copper binding ligands. This therefore confirmed that there must be two distinct forms of metallothioneins in both *Portunus pelagicus* and *Scylla serrata*.

Lerch (22) had shown that the two apothioneins from *Scylla serrata* have very similar amino acid compositions. Using ^{1}H NMR studies, Galdes and Hill (24) had previously deduced that the structure of the apothioneins is either purely random-coil or sufficiently loose such that each residue experiences the same environment. This suggests that the apothioneins do not possess well-defined tertiary structures in the conditions under which NMR analysis has been carried out. Under the unphysiological reversed phase chromatography conditions, it is possible that the molecules lose, at least partially, their tertiary structures which would expose internal residues. However the vast difference in the hydrophobicities of two proteins with such similar amino acid

sequence (in the case of the metallothioneins from *Scylla serrata*) indicates that the two protein molecules may be conformationally different and that under the chromatographic conditions used for their analysis, they retain, at least partially their conformations in solution.

In conclusion, present studies confirmed that there are two forms of metallothionein in *Scylla serrata* and *Portunus pelagicus* and demonstrated the usefulness of reversed phase HPLC as a method for the purification of these proteins.

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