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CHARACTERISTICS OF SCORPIONFISH METALLOTHIONEINS

This study confirmed that the substance to which metals are bound in the so-called metallothionein-containing pool of scorpionfish (Brown *et al.* this report) is indeed metallothionein. This was done by first isolating and purifying the metal-binding components of this pool and then showing that these components had the properties of metallothionein including its unique amino acid composition.

Some 20% of the amino acids of metallothionein are cysteine; it contains no aromatic amino acids. This amino acid composition can be used to confirm metallothionein since the amino acid composition and sequence are quite similar for metallothioneins isolated from the various phyla. Apparently this protein evolved before the development of diverse life forms and its structure has been conserved by evolutionary selection (Kagi and Nordberg 1979; Learch *et al.* 1982).

In our detoxification studies, we have taken advantage of the relatively small size of metallothioneins by using gel permeation chromatography to separate them from metal-containing proteins that have a molecular weight greater than 25,000 daltons and from metals that are associated with compounds such as amino acids which have a molecular weight less than 1,000 daltons. As can be seen in Figure 1, this procedure results in a profile with up to 3 discrete metal-containing peaks with the intermediate peak (II) containing metallothioneins. This procedure separates molecules only on the basis of molecular weight, but the metallothionein pool also contains other cytosolic molecules falling in the 10-12000 molecular weight range. Note that although metallothionein has an actual molecular weight of 6000-7000 its oblong shape makes it behave as a larger molecule in gel-permeation chromatography; thus, the observed molecular weight of 10-12000 daltons.

In this study we examined the metallothionein-containing pool obtained by gel permeation chromatography and characterized it in terms of the number and types of proteins, as well as the number of metal-binding proteins it contains. We then examined the metal-binding proteins of this pool on a structural and functional basis and compared them with known metallothioneins.

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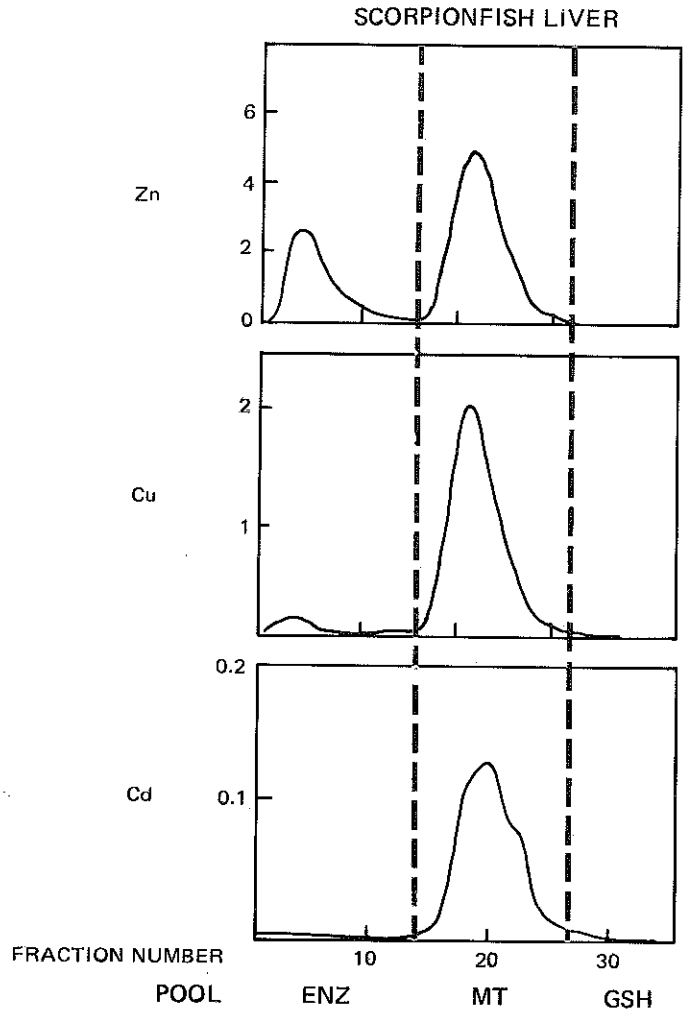

Figure 1. Sephadex G-75 profile showing the concentration of Zn, Cu and Cd in individual fractions of the enzyme-containing pool (ENZ), the metallothionein-containing pool (MT) and the glutathione-containing pool (GSH).

METHODS

Scorpionfish were collected by small otter trawl from Dana Point. Animals were sacrificed, livers removed, and cytosols prepared as described previously (Brown *et al.* this report) and stored at -80°C until analyzed.

In order to expedite the detection of metallothioneins, during isolation procedures ^{109}Cd was added to aliquots of the cytosol to a final concentration of 0.1 microcuries ^{109}Cd/ml and this was incubated at 4°C for 10 min since Cd readily displaces Zn from metallothioneins. This provides a specific and effective way to tag and trace metallothioneins. Cadmium labeled samples were counted in a Beckman gamma counter with 75% efficiency.

For Sephadex G-75 chromatography, cytosolic aliquots were thawed, vortexed, and 7 ml applied to a 1.6 x 70 cm column. Samples were eluted with 0.05 M Tris-HCℓ (pH 8.2) at a flow rate of 28.0 ml/hour and 3 ml fractions were collected. For HPLC, one ml aliquots of the

^{109}Cd labeled cytosols were chromatographed on a TSK SW 3000 stearic-exclusion column (Toya Soda) with 0.25M Tris HCℓ, pH 7.4 at a flow rate of one ml/min. The elutant was monitored at 250 nm and one ml fractions were collected. Pooled metallothionein fractions from the G-75 elutant were added directly to a 1.6 x 20 cm column packed with DEAE-Sephadex A-25 (Pharmacia) and eluted with a 300 ml gradient of 0.02-0.6 M Tris-HCℓ pH 7.4.

Peaks tentatively identified as metallothionein-containing were checked for purity using polyacrylamide gel electrophoresis after both Sephadex G-75 gel chromatography and DEAE-Sephadex A -25 chromatography.

Amino acid composition of the DEAE A-25 purified metal binding proteins was determined using a Beckman automated amino acid analyzer.

RESULTS AND DISCUSSION

Characterization of the Sephadex G-75 Metallothionein Pool:

Sephadex G-75 profiles of the scorpionfish cytosol (Figure 1) resulted in a metallothionein pool which contained significant proportion of the total cytosolic Zn (68%), Cu (95%), and Cd (95%). *In vitro* labeling of the cytosol resulted in a similar pattern with some 96% of the total ^{109}Cd associated with the metallothionein pool, presumably as a consequence of Zn displacement. The metal ratio in the scorpionfish metallothionein pool is Zn 1, Cu 0.62, and Cd 0.29. In terms of Zn and Cu, this ratio is similar to that reported for other fish (Noel-Lambot *et al.* 1978), but the Cd content is somewhat higher. This may reflect elevated Cd exposures in the fish from the Southern California Bight relative to those from Noel-Lambot's study (Jenkins *et al.* 1982). The effectiveness of these proteins in sequestering ^{109}Cd (98% bound in 10 min. at 4°C) is very suggestive of metallothioneins.

In terms of size, the scorpionfish metal-binding proteins have an apparent molecular weight of 10,000-11,000 as determined by both Sephadex G-75 and SW 3000 (HPLC) chromatography and comigrate on both columns with metallothioneins isolated from mouse livers (Figures 2a and b). These facts tend to strongly support the suggestion that these metal-binding proteins are metallothionein.

Rechromatography of combined fractions from the G-75 metallothionein pool results in a single peak on HPLC as determined by absorbance at 250 nm and ^{109}Cd activity suggesting the possibility of a pure metal-binding protein. However, HPLC separation, like G-75 chromatography, is based primarily on molecular weight and is not an absolute indicator of purity. Therefore, polyacrylamide gel electrophoresis which separates on the basis of both size and charge of the molecule was conducted. This revealed that approximately a dozen proteins were present in this partially purified pool. Over 95% of the bound ^{109}Cd activity was found associated with one or two lightly staining bands. These observations suggest that, although the metallothionein pool from Sephadex G-75 contains a number of proteins, most of the Cd is bound to only two of these proteins. Ion exchange chromatography shows that these proteins probably correspond to Metallothionein I and II, the two known forms of metallothionein (Kagi and Nordberg 1979).

Purification and Confirmation of Metallothionein

In order to purify large amounts of the metal-containing proteins from the metallothionein pool for amino acid analysis, samples from the G-75 elutant were combined and further frac-

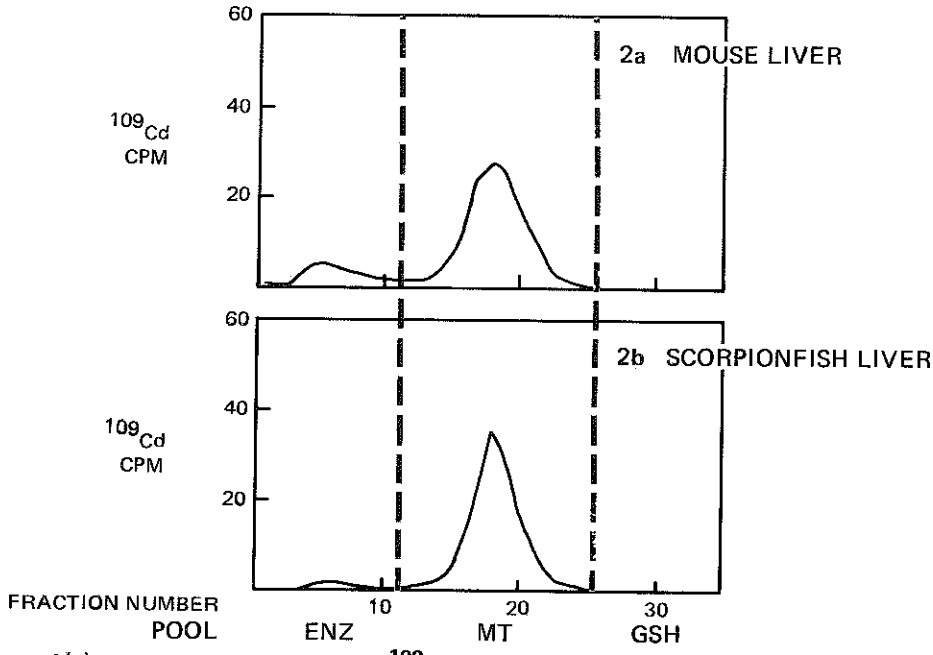

Figure 2(a). Sephadex G-75 profile of ^{109}Cd loaded metallothionein from scorpionfish liver cytosol (b). Sephadex G-75 profile of ^{109}Cd loaded metallothionein from mouse liver cytosol run under the same conditions. CMP: counts per minute x 10^{-3}.

tionated by an ion exchange chromatography on DEAE-Sephadex A-25. The resulting metal profile is shown in Figure 3. As can be seen, the ^{109}Cd is found in two discrete peaks which elute at 0.25 M and 0.35 M Tris-HCℓ buffer. These metal-containing peaks elute at molarities corresponding well with the two isomers of metallothionein, Metallothionein I and Metallothionein II, which have been identified in a number of vertebrate species including several fish species (Noel-Lambot *et al.* 1978; Olafson and Thompson 1974; Overnell and Coombs 1979). The presence of over 90% of the total cadmium in these two peaks suggests that they represent the major metal binding proteins of the initial metallothionein pool. Gel electrophoresis revealed that each of these peaks was a pure protein. The small peak eluting prior to gradient fractions 5-9 did not absorb strongly in the 250-300 nm range and had a low 250/280 ratio suggesting that it was not an intact metallothionein. The presence of some Cd in this small peak suggests that not all metal in the metallothionein-containing pool is bound to metallothionein. Therefore, only the two major metal-binding peaks tentatively identified as Metallothionein I and II, and shown to consist of one type of protein each, were taken for amino acid analysis.

CONCLUSION

As can be seen in Table 1, the amino acid content of scorpionfish metal-binding proteins, tentatively identified as Metallothionein I and Metallothionein II, shows both a high cysteine content and a lack of aromatic amino acids. This composition agrees quite well with those found in metallothioneins isolated from livers of other fish species (Noel-Lambot *et al.* 1978 and Overnell and Coombs 1979), as well as metallothioneins isolated from mammalian tissues (Kagi and Nordberg 1979). Taken together these data indicate that the major metal binding proteins of the metallothionein pool are indeed metallothioneins.

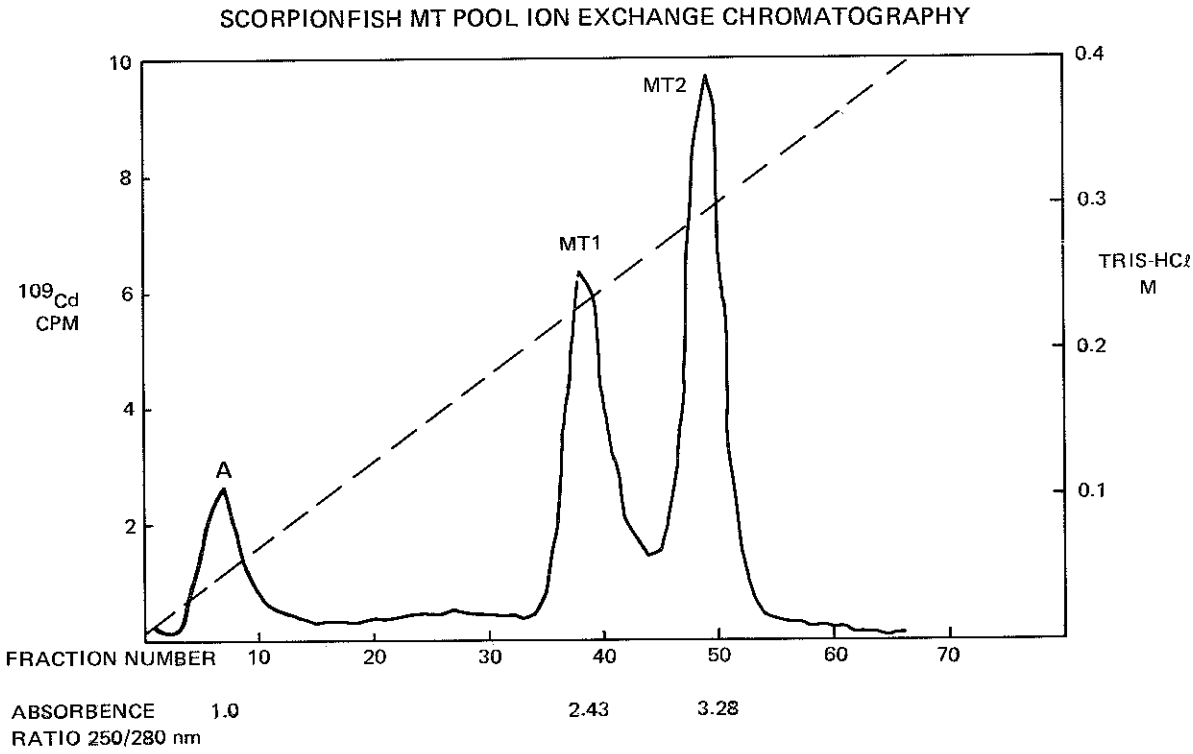

Figure 3. Ion exchange chromatography (DEAE Sephadex A-25) of the pooled MT fraction from a Sephadex G-75 separation. MT a and MT 2 elute at a molarity (M) of Tris-CH1 (pH 7.4) that is characteristic of known metallothioneins and with a 250/280 nm absorbence ratio also characteristic of metallothionein. MT1 = metallothionein 1 and MT 2 = metallothionein 2. CPM: Counts per minute x 10^3.

Table 1. This is the amino acid composition of metal-binding proteins from scorpionfish confirmed as metallothionein. Metallothioneins are characterized by a high cysteine content (20%) and no aromatic amino acids (marked below with an asterisk). Aromatic amino acids are those which contain a benzene ring.

Amino Acid	Percentage of Composition Metallothionein I	Metallothionein II
Cysteine	19.5%	25.4%
Serine	9.8	10.5
Lysine	8.5	8.4
Alanine	3.8	3.5
Glycine	11.9	12.4
Aspartic	9.9	11.8
Threonine	9.5	11.9
Glutamic	4.4	4.2
Proline	2.5	4.0
Valine	2.5	2.3
Methionine	3.5	2.0
Isoleucine	4.4	0.9
Leucine	3.6	0.9
Arginine	1.1	0
Histidine	1.5	1.9
Phenylalanine*	1.7	0
Tyrosine*	1.6	0
Tryptophan*	0	0

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