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Isolation of novel metal-binding proteins distinct from metallothionein from spotted seatrout (*Cynoscion nebulosus*) and Atlantic croaker (*Micropogonias undulatus*) ovaries

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Abstract. Metal-binding proteins were isolated from ovaries of the spotted seatrout *Cynoscion nebulosus* and the Atlantic croaker *Micropogonias undulatus* collected in 1988 near Port Aransas, Texas, USA. Gel-filtration analysis of spotted seatrout trout ovarian cytosolic fraction on Sephadex G-75 revealed the presence of three zinc-binding protein fractions. A major zinc/calcium-binding protein fraction had a low molecular weight (M_r) (6 000 to 10 000), similar to mammalian hepatic metallothionein (MT). All the metals were displaced from this fraction following saturation with exogenous cadmium. After exposure of Atlantic croaker to 2 mg cadmium l^{-1} seawater for 2 mo, the majority of the cadmium in the ovarian cytosolic fraction was associated with a similar low molecular weight protein fraction. These proteins were further purified by heat treatment and sequential acetone precipitation. Three isoforms were isolated by reverse-phase high-performance liquid chromatography. All the isoforms were found to be distinct from mammalian MT, based on amino acid composition. The major isoform contained low amounts of cysteine (approximately 5 residues per molecule) and aromatic amino acids, compared to high amounts of cysteine (typically 17 to 20 residues/molecule) and a lack of aromatic amino acids for mammalian MT. All the ovarian protein isoforms contained more glutamate than mammalian MT. The spotted seatrout and Atlantic croaker ovarian isoforms showed a high degree of homology with metal-binding proteins isolated from mammalian gonadal tissues. The results suggest a physiological role for these metal-binding proteins in developing vertebrate ovaries as well as an involvement in the sequestration of cadmium following environmental exposure.

Introduction

A variety of low molecular weight metal-binding proteins, both metallothioneins (MT) and non-metallothioneins, have been isolated from a broad range of species including vertebrates, invertebrates, plants and microorganisms (reviewed in Stone and Overnell 1985, Hamer 1986). MTs are low molecular weight (M_r) intracellular proteins that exhibit high affinities for heavy metals such as cadmium and mercury and are considered to be involved in the detoxification of these metals (Day et al. 1984, Goering and Klaassen 1984). Mammalian MTs have high levels of cysteine (approx 33%), lack aromatic amino acids, have high metal contents (4 to 12 nmol metal $nmol^{-1}$ protein), low M_r (6 000 to 10 000), and are heat-stable (Kagi et al. 1974, Kagi and Nordberg 1979).

MT is thought to have an important role in the development of tolerance to heavy metal toxicity. Tolerance has been demonstrated towards cadmium-induced lethality (Probst et al. 1977, Baer and Benson 1987), inhibition of hepatic drug metabolism (Roberts and Schnell 1982), hepatic necrosis (Morita 1984) and teratogenicity (Layton and Ferm 1980). MT may function in the homeostasis of essential heavy metals, specifically, zinc and copper (Reviewed in Cousins 1985). Zinc-MT levels are increased in tissues undergoing rapid development and differentiation such as the neonatal rat liver (Wong and Klaassen 1979). Since zinc is an important cofactor for nucleic acid synthesis (Rubin 1972), MT may be involved in the regulation of DNA synthesis (Ohtake et al. 1978, Ohtake and Koga 1979). The ability of zinc-MT to transfer zinc to metal-dependent enzymes has been demonstrated (Udom and Brady 1980). These results suggest that MT serves as a zinc donor during periods of high metabolic demand for zinc.

Metal-binding proteins have been isolated from sea urchin eggs (Ohtake et al. 1983), rat testes (Waalkes and Perantoni 1986), patas monkey testes (Waalkes et al. 1988a) and hamster ovaries (Waalkes et al. 1988b). These proteins are distinct from MT based on their ami-

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no acid compositions (i.e., low cysteine contents and the presence of aromatic amino acids). MT was not found in any of these tissues. Rat testes and hamster ovaries are extremely sensitive to cadmium-induced necrosis (Gunn and Gould 1970, Rehm and Waalkes 1988). These findings suggest a relationship between the extreme toxicity of cadmium and MT deficiency in these tissues.

It is not known whether metal-binding proteins are present in the gonads of other vertebrate groups, including teleosts, and whether they interact with zinc and zinc-requiring enzymes. Furthermore, the mechanisms by which toxic metals interact with metalloproteins and other cellular constituents in vertebrate gonads remain to be elucidated. The purpose of the present investigation was to determine whether metal-binding proteins are present in the ovaries of two marine teleosts belonging to the family Sciaenidae, spotted seatrout (*Cynoscion nebulosus*) and Atlantic croaker (*Micropogonias undulatus*). The homology between these metal-binding proteins and metal-binding proteins previously isolated from mammalian gonadal tissues was also examined.

Materials and methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) unless otherwise noted. Cadmium chloride ($CdCl_2$) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Acetone and acetonitrile were obtained from American Scientific Products (Houston, Texas, USA).

Fishes and sample preparation

Adult female spotted seatrout (*Cynoscion nebulosus*) and Atlantic croaker (*Micropogonias undulatus*) were captured by gill netting in shallow bays in the vicinity of Port Aransas, Texas, in 1988, transported to the laboratory, and maintained in 12 000-liter tanks supplied with running seawater at ambient temperature (24° to 27 °C) and salinity (30 to 35‰). Fish were kept in a 14 h light:10 h dark cycle and fed chopped liver and shrimp. In a separate experiment, Atlantic croaker were exposed to 2 mg cadmium l^{-1} seawater (30 to 35‰ S) for 2 mo. Fish with fully grown ovaries were anesthetized with quinaldine sulfate (Argent) and their spinal cords were severed. Ovaries were rapidly excised, rinsed, weighed and either used immediately, or stored at –80 °C. Tissues were homogenized (1:4, w/v) in 20 m*M* tris-HCl (pH 8.6) containing 5 m*M* 2-mercaptoethanol and 250 m*M* sucrose, using a Wheaton glass homogenizer and a motor-driven Teflon pestle. All procedures were carried out at 0° to 4 °C. A cytosolic fraction was obtained by successive centrifugation at 10 000 × *g* for 10 min and 100 000 × *g* for 60 min. The ovarian cytosolic fraction was stored at –80 °C prior to gel-filtration chromatography or further purification by heat treatment and sequential acetone precipitation. No differences in the elution profiles on gel-filtration between fresh and frozen tissues were observed.

The ovarian metal-binding protein was further purified by the method of Sobocinski et al. (1978) for the partial purification of MT from hepatic cytosol. This method has also been used to purify cadmium-binding proteins from rat and monkey testes (Waalkes and Perantoni 1986, Waalkes et al. 1988 a), and hamster ovaries (Waalkes et al. 1988 b). An ovarian cytosolic fraction was subjected to heat treatment (85 °C for 10 min) and centrifuged at 27 000 × *g* for 15 min. The heat-stable protein was then subjected to selective acetone precipitation (0 to 40%, 40 to 60%, 60 to 80% acetone by volume) with centrifugation (27 000 × *g* for 15 min) between each step. The 80% acetone precipitate was washed in acetone and stored under nitrogen until reconstitution in: (a) 20 m*M* tris/HCl pH 8.6, containing 5 m*M* 2-mercaptoethanol for anion-exchange high-performance liquid chromatography (HPLC); or (b) 0.1% trifluoroacetic acid containing 3 *M* guanidine hydrochloride for reverse-phase HPLC.

Gel-filtration chromatography

Spotted seatrout ovarian cytosolic fractions were either prepared for gel-filtration as described previously, or saturated with cadmium by the addition of 1.33 μmol cadmium g^{-1} wet wt of tissue equivalent. This cadmium dose saturates the zinc-binding protein from ovarian cytosolic fractions (own unpublished observations). Atlantic croaker ovarian cytosolic fractions were prepared following in vivo exposure to cadmium. Samples were loaded onto a Sephadex G-75 column (1.7 × 100 cm) equilibrated with 20 m*M* tris/HCl, pH 8.2, containing 20 m*M* KCl and 5 m*M* 2-mercaptoethanol. The column was eluted with the same buffer at a flow rate of 24 ml h^{-1} at 0° to 4 °C. Fractions (2.5 ml) were collected and directly analyzed for zinc, cadmium, calcium, magnesium, copper and iron by atomic absorption spectrophotometry (AAS) (Perkin Elmer, Model 360). Column void-volume (V_0, 65 ml) was determined using blue dextran.

Anion-exchange HPLC

Samples of the 80% acetone precipitate from heat-treated spotted seatrout ovarian cytosol were reconstituted in 2.0 ml of buffer g^{-1} wet wt equivalent and then saturated with cadmium. An aliquot was applied to a Waters 600 E HPLC equipped with a Protein PAK DEAE-5PW column (0.75 × 7.5 cm) equilibrated with 20 m*M* tris-HCl, pH 8.6, containing 5 m*M* 2-mercaptoethanol. The column was developed with a linear gradient from 1 to 200 m*M* NaCl at a flow rate of 42 ml h^{-1} at ambient temperature. Fractions (1 ml) were collected and analyzed for cadmium and protein content. Protein contents of chromatographic fractions were estimated by a modified Bradford protein assay with bovine serum albumin as the standard. The number of cadmium-binding sites in the ovarian protein was estimated by measuring the ratio of cadmium to protein content.

M_r estimation

The M_r of the major metal-binding protein fraction from spotted seatrout ovaries isolated by anion-exchange HPLC was estimated using gel-permeation HPLC. This technique is also used to assess the homogeneity of the protein (Ohtake et al. 1983). Samples were applied to a Waters Model 600 E system controller and multisolvent delivery system equipped with a SOTAPhase GF200 column (0.71 × 30 cm). The column was equilibrated with 250 m*M* tris-HCl, pH 7.2, and eluted with the same buffer at a flow rate of 24 ml h^{-1} at ambient temperature. The absorbance of the eluant was monitored at 230 nm with a SpectroMonitor III detector (Laboratory Data Control). The following protein standards were used for column calibration: blue dextran (M_r, 2 000 000), albumin (66 000), carbonic anhydrase (29 000), myoglobin (16 900), rabbit liver MT (apparent molecular weight 10 000), aprotinin (6500), and tryptophan (204.2).

Reverse-phase HPLC

The reverse-phase HPLC method developed by Klauser et al. (1983) to isolate MT isoforms was used for the isolation of ovarian metal-

binding protein isoforms. Samples of the 80% acetone precipitate from spotted seatrout and Atlantic croaker heat-treated cytosols were reconstituted in 2.0 ml of buffer g^{-1} wet wt equivalent and applied to a Zorbax Bio Series Protein Plus column (0.46 × 25 cm) (Dupont, Wilmington, Delaware, USA) with a 65 min linear gradient of 25 to 40% acetonitrile in 0.1% trifluoroacetic acid. Flow rate was adjusted to 1.0 ml min^{-1}, the absorbance was monitored at 215 nm, and 1 ml fractions were collected. This method caused a loss of metal-binding capacity in the protein samples.

Amino acid analysis and partial sequence

For amino acid analysis, fractions containing the separate isoforms of spotted seatrout and Atlantic croaker ovarian proteins after reverse-phase HPLC were divided into two aliquots and freeze-dried. One sample was hydrolysed at 110 °C with 6 *N* HCl for 22 h, while the other aliquot was first oxidized in performic acid for 4 h at 4 °C, diluted with water, freeze-dried and hydrolysed as before. The amino acid composition was determined on a Beckman 121MB amino acid analyzer (this procedure underestimates tryptophan). In addition, a partial amino acid sequence of the major spotted seatrout ovarian isoform protein was obtained with an Applied Biosystems 477A Protein Sequencer.

Results

The Sephadex G-75 elution profiles of unsaturated and cadmium-saturated cytosolic fractions from *Cynoscion nebulosus* ovary are shown in Fig. 1. In the unsaturated elution profile, the metals eluted as four major peaks; the first peak eluted with a high M_r protein fraction (> 75 000), the second peak eluted in an intermediate M_r protein fraction (10 000 to 30 000), the third peak eluted with the fractions characteristics of MT (M_r = 10 000 to 15 000; relative elution volume, $V_e:V_0$ = 1.8 to 2.1, where V_e is elution volume of peak and V_0 is void volume), and a fourth peak eluted in a very low M_r fraction (< 5000). The fourth peak may represent a degradation product of a high M_r protein or a metal bound to very low M_r constituents such as glutathione or cysteine. The first peak contained zinc, calcium and magnesium, the second peak zinc and magnesium, the third peak zinc, calcium and small amounts of magnesium, and the fourth peak magnesium. No copper was detected in any of these peaks (limit of detection < 50 μg Cu l^{-1}). Exogenous cadmium added prior to chromatography became associated with the second, third and fourth peaks. Cadmium displaced zinc, calcium and magnesium from the third peak.

The elution profile of ovarian cytosolic fractions from *Micropogonias undulatus* exposed to 2 mg cadmium l^{-1} for 2 mo is shown in Fig. 2. The majority of the cadmium was associated with the MT fraction with a $V_e:V_0$ of 1.8 to 2.1. This indicates that the ovarian cytosol sequesters environmental cadmium in a MT-like fraction. In untreated samples this fraction contained zinc which could be displaced with exogenous cadmium (own unpublished observations). A small peak in the high M_r region was also observed.

The spotted seatrout ovarian protein (M_r = 6678) was further purified by heat treatment of the ovarian cytosol followed by sequential acetone precipitation. Most of the low M_r metal-binding protein (approx 90%) was found in

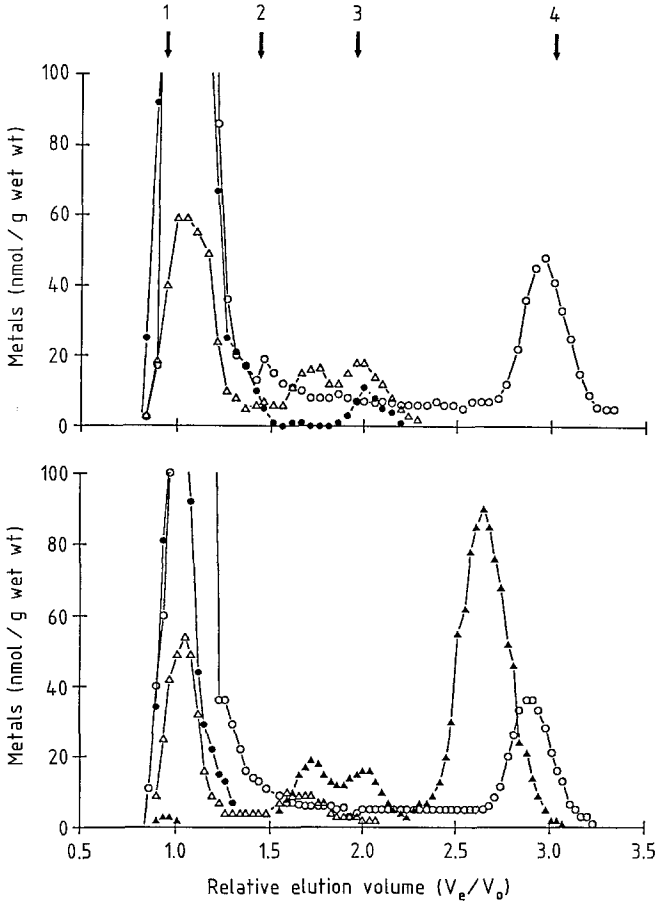

Fig. 1. *Cynoscion nebulosus.* Representative gel-filtration elution profiles of unsaturated and cadmium-saturated ovarian cytosolic fractions from spotted seatrout. Unsaturated cytosolic fraction (top graph) and cadmium-saturated cytosolic fraction (bottom graph) were applied to a Sephadex G-75 column and eluted with 20 m*M* tris-HCl (pH 8.6) containing 5 m*M* 2-mercaptoethanol at a flow rate of 24 ml h^{-1}. Fractions (3 ml) were collected and assayed for metals by atomic absorption. △: Zn; ▲: Cd; ●: Ca; ○: Mg; Cu and Fe were not detected. Elution positions of protein standards are shown: 1, blue dextran (M_r, 2 000 000); 2, carbonic anhydrase (29 000); 3, rabbit liver metallothionein (MT) standard (10 000); 4, aprotinin (6 500). Relative elution volume is defined as V_e/V_0, where V_e is elution volume of protein peak and V_0 is void volume

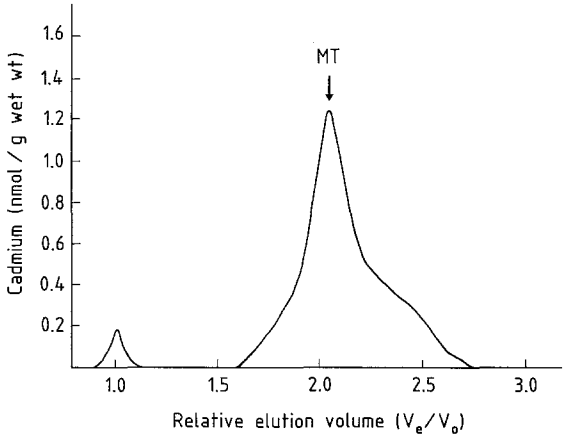

Fig. 2. *Micropogonias undulatus.* Representative gel-filtration elution profile of ovarian cytosolic fraction from Atlantic croaker following exposure to 2 mg cadmium l^{-1} seawater for 2 mo. Conditions identical to those described in legend to Fig. 1. MT: elution position of rabbit metallothionein

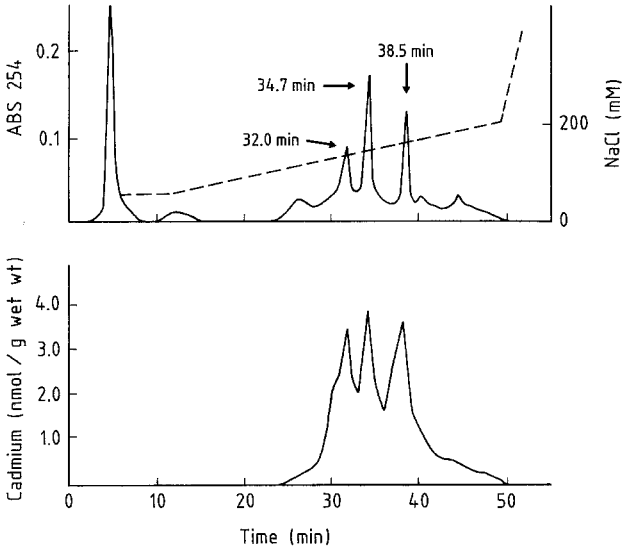

Fig. 3. *Cynoscion nebulosus.* Representative anion-exchange HPLC elution profile of ovarian protein from spotted seatrout following partial purification by heat-treatment and acetone precipitation. The 80% precipitate was saturated with exogenous cadmium as described in "Results" and applied to a DEAE-5PW column equilibrated with 20 m*M* tris-HCl (pH 8.6) containing 5 m*M* 2-mercaptoethanol. Column was developed with a linear gradient of NaCl (0 to 200 m*M*; dashed line) at a flow rate of 42 ml h^{-1}. Upper graph represents absorbance at 254 nm (CD-mercaptide bond), bottom graph represents cadmium as determined by atomic absorption

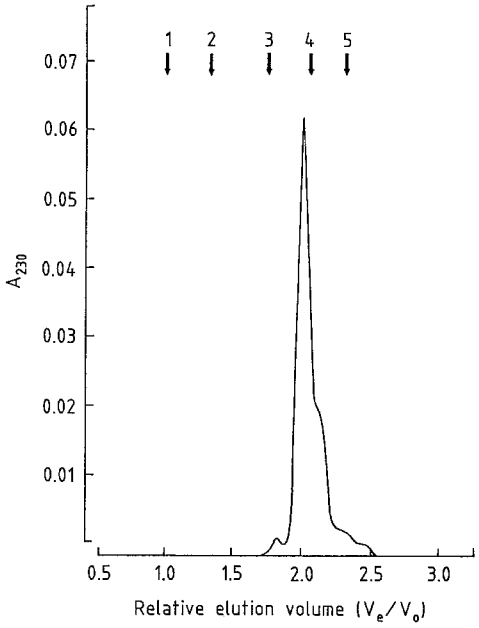

Fig. 4. *Cynoscion nebulosus.* Representative gel-permeation HPLC of the major ovarian metal-binding protein from spotted seatrout, purified by anion-exchange HPLC. Major metal-binding protein isolated from anion-exchange chromatography was applied to SOTAPhase GF200 column and eluted with 250 m*M* tris-HCl (pH 7.2), at a flow rate of 24 ml h^{-1}. Absorbance was monitored at 230 nm. Elution positions of the protein standards are shown: 1, blue dextran; 2, albumin (66 000); 3, rabbit liver MT standard; 4, aprotinin; 5, tryptophan (204.2)

the 80% acetone fraction (results not shown). The recovery of the protein by this extraction technique, measured by the ratio of the cadmium-binding capacity in cytosol to that in the heat-treated 80% acetone precipitate fraction, was approximately 97%. The 80% acetone precipitate fraction was saturated with exogenous cadmium, reconstituted in 2 ml buffer g^{-1} wet wt, and chromatographed on an anion-exchange column (Fig. 3). Three cadmium peaks were present (protein contents not shown) eluting at 32, 34.7 and 38.5 min. In separate experiments, the zinc contents of the three peaks before cadmium saturation were estimated to be 1.63, 3.63 and 1.91 mol zinc mol^{-1} protein, respectively, based on the M_r derived from amino acid analysis (see Table 1).

The major metal-binding protein fraction from spotted seatrout ovaries eluting at 34.7 min from anion-exchange HPLC was applied to a gel-permeation HPLC. One major peak and several minor peaks were observed at 230 nm (Fig. 4). The M_r was estimated to be about 6 600. From the elution profile on the gel-permeation column, the homogeneity of the protein was estimated to be 90% or greater. In addition, sodium dodecyl sulfate polyacrylamide-gel electrophoresis (a combination of 10 and 20% gels) of the protein showed one visible band following silver-staining and the M_r was estimated at 6 900 (results not shown).

The 80% acetone precipitate prepared from the spotted seatrout ovarian cytosolic fraction was also purified by reverse-phase HPLC (Fig. 5). The ovarian protein was again separated into three major isoforms eluting at 54.3, 56.0 and 59.5 min. The major isoform isolated by anion-exchange HPLC (34.7 min, see Fig. 4), was almost identical to the isoform eluting at 56.0 min based on amino acid

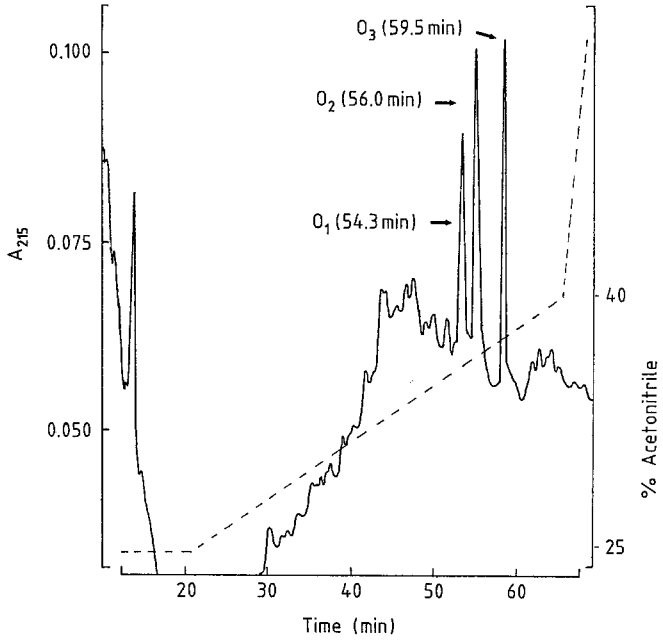

Fig. 5. *Cynoscion nebulosus.* Representative reverse-phase HPLC elution profile of ovarian protein from spotted seatrout following partial purification by heat-treatment and acetone precipitation. The 80% precipitate was satured with exogenous cadmium as described in "Results" and applied to a Zorbax Bio Series Protein Plus column with a 65 min linear gradient (25 to 40% acetonitrile) at a flow rate of 1.0 ml^{-1}. Fractions were monitored at 215 nm. Three major peaks, termed O_1, O_2 and O_3 by order of elution, were detected. Peaks prior to 20 min were due to buffer constituents

composition (results not shown). Both isoforms eluted second in their respective chromatograms. In another study, three ovarian isoforms from Atlantic croaker were isolated by HPLC and eluted at 47.8, 55.5 and 61.5 min, the major isoform eluting in the first peak (results not shown).

Amino acid analyses of spotted seatrout and Syrian hamster ovarian metal-binding proteins are shown in Table 1. Amino acid analyses of Atlantic croaker ovarian metal-binding proteins and rabbit hepatic MT-I are shown in Table 2. Marked differences are evident, especially in the cysteine contents, which are considerably lower in the spotted seatrout, Atlantic croaker and hamster ovarian proteins (2 to 7 residues per molecule in all cases) than in the rabbit hepatic MT-I (20 residues per molecule). Rabbit liver MT-I also exhibited higher serine and lysine contents (8 residues per molecule), whereas the spotted seatrout and Atlantic croaker ovarian proteins contained more isoleucine and leucine (2 to 4 and 4 to 6 residues per molecule, respectively) as well as significant amounts of phenylalanine and histidine (1 to 3 residues per molecule in all cases). The proteins from the spotted seatrout, Atlantic croaker and hamster ovaries also contained more glutamate (8 to 11 residues per molecule mol) than rabbit hepatic MT-I (3 residues per molecule).

A partial N-terminal amino acid sequence of the major spotted seatrout ovarian protein (termed O_3 by order of elution) is shown in Table 3. The N-terminal amino acid for the seatrout ovarian protein O_3 was lysine.

Discussion

The results of the present study indicate that the low M_r metal-binding proteins in the cytosolic fractions of *Cynoscion nebulosus* and *Micropogonias undulatus* ovaries are not MT. These proteins had many character-

Table 1. *Cynoscion nebulosus*. Amino acid compositions of spotted seatrout and Syrian hamster ovarian metal-binding proteins (composition of Syrian hamster protein from Waalkes et al. 1988b); analyzed after protein hydrolysis with 6 *N* HCl at 110 °C for 24 h. Isoforms (O_1, O_2, O_3) are defined by order of elution on reverse-phase high-performance liquid chromatography (Fig. 5). Data are expressed as number of residues per molecule; nearest whole number is shown in parentheses; compositions are given relative to alanine content

Amino acid	Seatrout ovarian proteins			Syrian hamster ovarian protein P_1
	O_1	O_2	O_3	
Aspartic acid	8.4 (8)	8.6 (9)	6.8 (7)	5.8 (6)
Threonine	1.9 (2)	4.3 (4)	4.6 (5)	3.7 (4)
Serine	4.7 (5)	4.0 (4)	7.4 (7)	6.1 (6)
Glutamic acid	9.4 (9)	9.2 (9)	10.3 (10)	10.7 (11)
Proline	2.6 (3)	2.4 (2)	1.0 (1)	3.4 (3)
Glycine	4.9 (5)	4.3 (4)	9.2 (9)	3.2 (3)
Alanine	2.9 (3)	3.2 (3)	4.3 (4)	4.0 (4)
Valine	3.3 (3)	3.4 (3)	2.1 (2)	0.92 (1)
Half-cystine[a]	3.6 (4)	3.1 (3)	4.8 (5)	5.2 (5)
Methionine	0.8 (1)	0.6 (1)	1.4 (1)	1.0 (1)
Isoleucine	3.6 (4)	3.9 (4)	2.1 (2)	1.8 (2)
Leucine	4.7 (5)	5.6 (6)	4.4 (4)	1.9 (2)
Tyrosine	0	0	0	0
Phenylalanine	2.4 (2)	1.9 (2)	2.2 (2)	1.0 (1)
Histidine	0.9 (1)	1.0 (1)	1.9 (2)	0
Lysine	4.3 (4)	4.4 (4)	1.1 (1)	8.9 (8)
Arginine	0.8 (1)	1.7 (2)	2.3 (2)	0.8 (1)
Total no. of residues	60	61	64	60
Min. molecular weight (M_r)	6478	6735	6678	6440

[a] Half-cystine was determined as cysteic acid after performic acid oxidation

Table 2. *Micropogonias undulatus*. Amino acid compositions of Atlantic croaker ovarian metal-binding proteins and rabbit hepatic metallothionein (obtained from Sigma Chemical Co.); analyzed after protein hydrolysis with 6 *N* HCl at 110 °C for 24 h. Isoforms (C_1, C_2, C_3) are defined by order of elution on reverse-phase high-performance liquid chromatography (results not shown). Data are expressed as number of residues per molecule; nearest whole number is shown in parentheses; compositions are given relative to alanine content

Amino acid	Croaker ovarian proteins			Rabbit hepatic metallothionein MT-I
	C_1	C_2	C_3	
Aspartic acid	5.7 (6)	7.9 (8)	5.8 (6)	4.8 (5)
Threonine	2.8 (3)	1.2 (1)	2.7 (3)	4.1 (4)
Serine	5.5 (6)	5.6 (6)	4.2 (4)	7.7 (8)
Glutamic acid	8.9 (9)	10.6 (11)	7.9 (8)	2.7 (3)
Proline	3.3 (3)	2.6 (3)	1.9 (2)	2.4 (2)
Glycine	4.2 (4)	6.3 (6)	4.5 (5)	4.0 (4)
Alanine	4.3 (4)	3.4 (3)	2.5 (3)	7.1 (7)
Valine	4.6 (5)	1.9 (2)	3.0 (3)	0
Half-cystine[a]	2.8 (3)	7.2 (7)	2.1 (2)	19.8 (20)
Methionine	0.9 (1)	0.7 (1)	0.5 (1)	1.0 (1)
Isoleucine	2.0 (2)	3.3 (3)	2.6 (3)	1.3 (1)
Leucine	5.3 (5)	4.9 (5)	4.3 (4)	0.6 (1)
Tyrosine	1.2 (1)	0	0.8 (1)	1.6 (2)
Phenylalanine	1.9 (2)	2.7 (3)	2.7 (3)	0
Histidine	1.3 (1)	0.8 (1)	0.5 (1)	0
Lysine	2.9 (3)	5.1 (5)	4.5 (5)	8.1 (8)
Arginine	2.8 (3)	1.4 (1)	0.9 (1)	1.0 (1)
Total no. of residues	61	66	55	67
Min. molecular weight (M_r)	6681	7136	6012	6799

[a] Half-cystine was determined as cysteic acid after performic acid oxidation

Table 3. *Cynoscion nebulosus*. Partial N-terminal amino acid sequence of the major spotted seatrout ovarian metal-binding protein (O_3 peak from reverse-phase HPLC, Fig. 5)

Lysine	Arginine	Glycine	Serine	Histidine	Valine	Histidine	Serine	Tryptophan	Threonine
1	2	3	4	5	6	7	8	9	10

istics of mammalian MT, such as a similar M_r and high metal content, but differed dramatically based on amino acid composition. For example, a major difference was observed in cysteine content, which is prominent in mammalian MT (approx 17 to 20 residues per molecule, Kagi et al. 1974), but was relatively low in the seatrout and Atlantic croaker ovarian proteins (approx 2 to 7 residues per molecule). The seatrout and Atlantic croaker ovarian proteins also had fewer serine and lysine residues compared to mammalian MT. The seatrout and Atlantic croaker ovarian proteins contained leucine, phenylalanine and histidine, all of which are typically absent from mammalian MT (Webb 1979). The glutamate content was also higher in the seatrout and Atlantic croaker ovarian proteins compared to mammalian MT. This is the first demonstration, to our knowledge, of low M_r metal-binding proteins in teleost ovaries.

The spotted seatrout and Atlantic croaker ovarian proteins are very similar to metal-binding proteins previously isolated from rat testes (Waalkes et al. 1984, Waalkes and Perantoni 1986), patas monkey testes (Waalkes et al. 1988 a) and Syrian hamster ovaries (Waalkes et al. 1988 b) based on amino acid composition. All of these gonadal proteins contained similar amounts of glutamate, cysteine and aromatic amino acids. Cornish-Bowden (1981) introduced a simple method that allows one to determine statistically whether two proteins are similar based on their amino acid composition. If two proteins are found by this method to be similar, there is a high possibility that their sequences are related. The method of Metzger et al. (1968) was also used to compare proteins that differed in their number of residues. Using these two methods, it was concluded that the spotted seatrout and Atlantic croaker ovarian proteins are related to the gonadal proteins mentioned above (proteins differed by $<42\%$) and are probably unrelated to mammalian MT (differences $> 93\%$). Thus, there may be a homology among gonadal metal-binding proteins in different species. Similarly, MT is a highly conserved protein in a variety of diverse species (Vasak and Armitage 1986).

The seatrout ovarian protein exhibited a relatively high metal content, approx 2 to 4 mol zinc mol^{-1} protein. Mammalian MT typically has 4 to 12 mol metals mol^{-1} protein and these metals are exclusively bound by clusters of thiolate bonds (reviewed in Hamer 1986). Since low amounts of cysteine were present in the seatrout ovarian protein, metal-binding to cysteine residues would be limited. Waalkes et al. (1988 a) discussed the possibility that amino acids containing carboxy groups, such as glutamate and aspartate, may be alternative binding sites for metals. Glutamate is the most prominent amino acid in the seatrout ovarian protein as well as in the mammalian gonadal proteins (Waalkes et al. 1984, Waalkes et al. 1988 a, b). In addition, aspartate is relatively high in the seatrout ovarian protein compared to mammalian MT. Jacobson and Turner (1980) demonstrated that zinc interacts with carboxy groups, but with less avidity than with cysteine. Carboxy groups are thought to provide secondary low-affinity binding sites for cadmium (Jacobson and Turner 1980).

The function of these ovarian proteins from the spotted seatrout and Atlantic croaker is uncertain. Numerous investigators have postulated a role for MT in the homeostatic regulation of essential metals such as zinc and copper (Wong and Klaassen 1979, Brady 1982). Brady and Webb (1981) found increases in zinc- and copper-MT in developing rat tissues and suggested that these high levels of MT were required for nucleic acid metabolism, protein synthesis and other metabolic processes in rapidly developing tissues. In mammalian testes, the demand for zinc is also high due to the high rates of DNA, RNA and protein synthesis, all of which require zinc (Halsted et al. 1974, Brady 1982). There is evidence that the testicular pool of zinc is very labile in comparison with other tissues (Halsted et al. 1974). The apparent low binding affinity of zinc for the testicular metal-binding proteins may provide a storage depot for zinc that may be rapidly mobilized and thus more responsive to changes in testicular zinc demand (Waalkes et al. 1988 a).

The ovarian protein of the spotted seatrout may have a similar function. The maturing oocyte is actively involved in the accumulation and production of proteins that are essential for nuclear DNA and protein synthesis in later stages of growth (Benbow 1985). During final oocyte maturation, germinal vesicle breakdown releases DNA polymerases (Forbes et al. 1983, Zierler et al. 1985). Similarly, Grippo et al. (1977) showed a dramatic increase in total DNA polymerase activity during final oocyte maturation. Slater et al. (1971) reported that zinc is an essential component in purified DNA polymerase from the nuclei of sea urchin blastulae, which suggests that the cellular demand for zinc may be high during final oocyte maturation. The initiation of nuclear DNA synthesis is an important and major functional change at the onset of embryogenesis. There is preliminary evidence in 12 h fertilized seatrout eggs of a decline in zinc associated with the protein, indicating a possible transfer of zinc to zinc-requiring enzymes (own unpublished observations). A role for the ovarian protein of the seatrout may be to provide a source of zinc in the maturing oocyte as well as during developmental stages following fertilization.

The function of the ovarian metal-binding proteins of the seatrout and croaker in metal detoxification, historically an important role ascribed to mammalian MT (Terhaar et al. 1965, Webb and Verschoyle 1976, Baer and Benson 1987), is not known. The apparent lack of MT in the rat and patas monkey testes (Waalkes and Perantoni 1986, Waalkes et al. 1988 a) and hamster ovaries (Waalkes et al. 1988 b) may be a reason for the sensitivity of these organs to cadmium toxicity. The testes develop pathological lesions in the presence of very low tissue-burdens of cadmium (Wong and Klaassen 1980). It is therefore possible that the testicular metal-binding protein isolated from that organ is not associated with metal detoxification.

The present investigation shows that the ovarian proteins in Atlantic croaker can sequester cadmium following environmental exposure. The major isoform in croaker after cadmium exposure eluted in the first protein peak (47.8 min), whereas the major isoform from untreated spotted seatrout eluted in the second peak

(56.0 min). It is not known whether these varied profiles reflect heavy metal exposure or merely species differences, since possible increased synthesis of any of these isoforms following cadmium exposure was not examined.

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