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First-time report of metalloproteinases in fish bile and their potential as bioindicators regarding environmental contamination

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ABSTRACT

Gallbladder bile from 2 fish species, mullet (*Mugil liza*) and tilapias (*Tilapia rendalli*), contain substantial matrix metalloproteinases (MMPs). Extensive purification studies were conducted in order to obtain workable samples for SDS-PAGE and zymography analysis. Proteinase activities were assayed by gelatin substrate zymography. Several protein bands were observed, corresponding to molecular weights of 200, 136, 43, 36, 34, 29, 23 and 14 kDa in mullet bile and 179, 97, 79, 61, 54, 45, 36, 33 and 21 kDa in tilapia bile. Specific inhibitor studies were conducted, in which MMPS were inhibited by EDTA and 1,10 phenanthroline, but not by serine and cysteine protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF) and *trans*epoxysuccinyl-L-leucylamido-L-guanidino butane (E-64), confirming the proteinase identities as MMPs. Differences in proteinase expression were observed in fish from a contaminated and reference site. Some studies regarding MMPs in different fish tissues exist, however this is the first study conducted in fish bile, and their involvement in detoxification processes and organism protection against the effects of aquatic contaminants may be a possibility.

1. Introduction

Fish have attracted considerable interest in studies assessing biological and biochemical responses to environmental contaminants (Powers, 1989). They are particularly useful for the assessment of water-borne and sediment-deposited toxins, providing advanced warning of the potential danger of the new chemicals and the possibility of environmental pollution. They are also particularly good models for studies involving biochemistry and comparative physiology, since they live in a variety of habitats and must adapt to environmental parameters and stress, easily reproduced in laboratory conditions (Powers, 1989). Thus, fish are well recognized bioindicators of environmental changes, including chemical pollution and the understanding of toxicant uptake, behavior and responses in fish, therefore, have a high ecological relevance. They are also an important link between the environment and human populations through fisheries and consumption by local and other markets (Espino, 2000; Food and Agriculture Organization of the United Nations, 1983).

Biliary excretion offers a way to analyze various contaminants in aquatic organisms, and fish bile has been used as a biomarker for environmental contamination. The rapid metabolism and elimination of several contaminants by vertebrates result in low residual concentrations of these contaminants in muscle and liver tissues (Galgani et al., 1992). Thus, chemical analysis of fish tissues has limited usefulness as an indicator of environmental exposure. In contrast, biliary excretion has been noted as an alternative indicator of exposure for environmental monitoring and screening purposes (Norris et al., 2000) i.e. habitats contaminated by polycyclic aromatic hydrocarbons (PAH), in which fish bile has shown high concentrations of biliary PAH metabolites (Ariese et al., 1993; Krahn et al., 1987) and metal exposure, since studies indicate that many metals are excreted from the liver to the bile (Bunton and Frazier, 1994; Dijkstra et al., 1996), such as copper (Grosell et al., 1998) and zinc (Handy, 1996).

Matrix metalloproteinases (MMPs) are a family of calcium-dependent, zinc-containing endopeptidase (Bode and Maskos, 2003). They breakdown extracellular matrix components and are part of both normal and pathological tissue remodeling and resorption, wound healing, embryo development, and tumor invasions (Matrisian, 1992; Woessner, 1994). Many different MMPs have been described in several species including plants, invertebrates, and many higher vertebrates (Massova et al., 1998), and are found in many different tissues, although their precise function in many

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of these tissues remains unclear (Rath et al., 2001). Notably, little information exists on MMPs in bile fluid, and studies regarding metalloproteinases in fish bile are non-existent. Therefore, it is of interest to conduct more studies on fish bile, since this may lead to other interesting discoveries in the environmental, proteomic and metalloproteomic fields.

The present study aims to characterize fish bile regarding their enzyme content, and to verify if these enzymes can be useful as a biomonitoring tool in relation to environmental contamination. *Mugil liza* (mullet) and *Tilapia rendalli* (tilapia) specimens were used for this characterization, since they are of economic interest in south-eastern Brazil, and are considered sentinel species for several compounds of environmental interest, such as metals and organic pollutants (Andres et al., 2000; Canli and Atli, 2003; Mansour and Sidky, 2003; Neves et al., 2007; Shah and Attinag, 2005). After the development of the sample clean-up the methodology was applied to environmental samples in order to verify bile MMP potential as biomarkers regarding environmental contamination, by comparing tilapia samples from a contaminated and reference site.

2. Materials and methods

2.1. Reagents and sample collection

The 2D Quant-Kit; albumin and IgG depletion columns and G-25 Sephadex desalting columns were purchased from GE Healthcare (Uppsala, Sweden), as well as all the electrophoretic reagents. All others were from Merck (Darmstaad, Germany). Proteinase inhibitors were purchased from Sigma Chemical Company (St. Louis, MO, USA). All solutions were prepared with deionized water ($\geq$18.2 MΩ cm) using a Milli-Q water purification system (Millipore, Bedford, USA).

Mullet (*M. liza*) and Tilapias (*T. rendalli*) were obtained fresh from two important fishing sites, Guanabara Bay and Rodrigo de Freitas Lagoon, located in the Rio de Janeiro state, Brazil. Both these contaminated sites are located in the urban Rio de Janeiro area, and are heavily contaminated by heavy metals and organic compounds such as polycyclic aromatic hydrocarbons (De Luca Rebello et al., 1986; Loureiro, 2004; Neves et al., 2007; Perin et al., 1997). The reference site commercializes fish for human consumption, in large tanks with controlled conditions. As recommended previously (Couture and Rajotte, 2003) sampling was conducted in order to yield a range of fish representative of the sampled population, using nonselective sampling methods (corrals, trawl nets, casting nets). A total of 8 mullet from Guanabara Bay and 15 tilapia (6 from the Rodrigo de Freitas Lagoon and 7 from the reference site) were sampled. Bile was collected by direct puncture of the gallbladder and immediately stored at −80 °C in sterile eppendorfs until analyses.

2.2. Sample purification protocols

The initial proposal was to identify metalloproteinase components in fish bile by one- and two-dimensional zymography of crude bile, in order to alter the samples as little as possible with clean-up steps. However, the first attempts at separating crude bile on one-dimensional gels revealed that fish bile fluid, as other organisms, contains high amounts of lipids and bile salts, interfering both in protein quantification and gel analysis. Therefore, six different clean-up protocols, based on previous human bile studies (Farina et al., 2009; Kristiansen et al., 2004) were analyzed, in order to obtain workable samples.

Protocol 1 consisted of simple centrifugation, in which 1 mL of crude bile was centrifuged at 16,000 × *g* for 10 min at 4 °C, using a Biofuge Stratos Centrifuge (Sorvall, USA).

Protocol 2 was a three-step lipid removal using Cleanascite™ HC, which is a non-ionic adsorbent, used to precipitate lipid fat droplets, cell debris and mucinous impurities. 1 mL of crude bile was centrifuged at 16,000 × *g* for 10 min at 4 °C, using a Biofuge Stratos Centrifuge (Sorvall, USA). 250 μL of the delipidizer Cleanascite™ HC (Ligo-Chem. Inc., Fairfield, NJ, USA) was added to the partially cleared bile supernatant, followed by sample rotation for 1 h at 4 °C. The sample was then centrifuged again at 16,000 × *g* for 10 min for lipid micelle removal.

Protocol 3 was composed of albumin and imunoglobulin G (IgG) depletion. Since these proteins are present in high quantities in several fluids, including bile, and interfere in many analyses, we decided to analyze the effects their depletion, using columns specific for albumin and IgG depletion (GE Healthcare, part number 28-9506-16 AB). These columns remove >95% of albumin and >90% IgG, and are highly specific for these two proteins, according to the manufacturer.

Protocol 4 was comprised of acetonitrile/ethanol (ACN/EtOH) protein precipitation, according to Osnes et al. (1993). Briefly, acetonitrile was added to bile samples in three steps, at 1/2, 1/2 and 3× the sample volume, followed by sample shaking and centrifugation. The final steps consisted in the addition of ethanol 96% (w/v), followed by sample shaking and centrifugation and then total evaporation of the samples under a gentle nitrogen flow. The samples were then resuspended in 120 μL 1.0 mmol L^{-1} tetrahydrate borate buffer, pH 11.

Protocols 5 and 6 focused on desalting: protocol 5 consisted of dialysis with a Spectra-Por® membrane with a 3.5 kDa molecular mass cut-off, using 1 mL of bile, performed against double-distilled water for 24 h, with several buffer changes throughout this period; and protocol 6 consisted of gel-filtration using Sephadex G-25 packed columns (GE Healthcare, part number 28-9225-30 AB), with a 5 kDa molecular mass cut-off, of 1 mL bile. These Sephadex columns are used for desalting and sample clean-up, where small molecules like salts, free labels and other impurities are efficiently separated from the high molecular weight substances of interest, in our case, bile proteins. The sample is diluted with a buffer of choice (usually ultra-pure water) and is passed through the column, yielding a salt-free sample.

Some of these clean-up steps were combined for further testing: after centrifugation, one group of samples was also delipided, and after delipidation, 2 groups of samples were also desalted by dialysis (one group) and gel-filtration (the other group) using the G-25 Sephadex desalting columns, as shown in the bile sample purification protocols and analysis flow (Fig. 1). SDS-PAGE separation of at least 3 crude and purified samples from each fish species (three individual fish from each species with no sample pooling conducted) indicated which protocol was adequate for further testing by gelatin zymography.

Interferents present in bile also result in difficult protein quantification (Osnes et al., 1993). Therefore, three different protein quantification methods were tested with crude and purified samples: the Lowry and Biuret methods and the GE Healthcare 2D Quant-Kit®. Data normality was tested using the Shapiro–Wilkes *W* test prior to additional statistical analyses

2.3. SDS-PAGE separation

15% Sodium dodecyl sulfate polyacrylamide gels were prepared as described previously (Laemmli, 1970). To determine appropriate zymography gel-loading, aliquots of fish bile as described above were analyzed by one-dimensional (1D) SDS-PAGE and stained by silver staining as described by Heukeshoven and Dernick (1985), with minor modifications. Subsequent refined zymography gel loading was determined based on the intensity and streaking of