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Proteome analysis of the fathead minnow (*Pimephales promelas*) reproductive testes

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ABSTRACT

Proteomics is becoming more widely used as a tool in fish physiology and toxicology and can offer mechanistic insight into organism responses to environmental signals and stressors. Using a LTQ Orbitrap Velos MS/MS, we detected 1075 proteins in the reproductive testis of fathead minnow. Proteins localized to the testis included those with a role in spermatogenesis, DNA repair, gamete meiosis, and proteins that have methylation and phosporylation activity, which are important regulatory mechanisms required for sperm maturation. Enrichment analysis revealed that proteins involved in translation, excision DNA repair, and chromatin remodeling were significantly enriched in the testis (>25% protein coverage of the cellular pathways). Proteins involved in RNA metabolism, spliceosome assembly, metabolism, and DNA unwinding were localized to the testis, and the DEAD (Asp-Glu-Ala-Asp) box RNA-dependent helicase family was well represented in this reproductive tissue. Based upon common detected proteins and functional processes between FHMs and the more ancient sharks, other ray-finned fishes, and mammals, we hypothesize that biological processes involved in the testis (DNA unwinding, RNA processing, spliceosome assembly) have been conserved throughout vertebrate evolution. This study provides the foundation for more in depth proteomics studies investigating the effects of hormones and endocrine disruptors in the teleostean testes.

1. Introduction

Studies that quantitate proteomic responses to aquatic pollutants, endogenous hormones, infection, and environmental stimuli have yielded novel insight into organismal physiology. Localization of proteins in a tissue, organelle, or cell type provides important mechanistic information about its function. In recent years, there have been tremendous technical advances in LC MS/MS based approaches and these methods are increasingly used to characterize proteomes in mammalian models. The proteome has been described for a range of mammalian tissues [1,2] as well as in cellular organelles [3], individual nuclei of the hypothalamus [4], and specific functional regions of tissues such as pancreatic islets [5] and olfactory cilia [6].

In addition to the aforementioned studies in mammals, proteomics has also become more accessible to include non-mammalian models used in a variety of biological disciplines. The proteome has been described for the goldfish (*Carassius auratus*) and fathead minnow (FHM) (*Pimephales promelas*) neuroendocrine brain [7–9], Cod (*Gadus morhua*) muscle [10], and FHM liver [11,12]. In zebrafish (*Danio rerio*), a model teleost used for studies in development and genetics, the proteome has been described for liver [13], whole brain [14], gill [15],

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caudal fin [16], and olfactory bulbs [17]. In addition to adult tissues, earlier life history stages of fish have been investigated for proteome content. Papakostas et al. [18] characterized the proteome in eyed and hatching stages of European grayling (*Thymallus thymallus*), functionally cataloging the proteins into those which are involved in the development of the central nervous system, skeleton, and heart. Thus, in teleost fish, there are now fundamental data about the composition of the proteome that can be used for more detailed studies regarding physiology and ecotoxicology.

In addition to non-reproductive tissues, studies have also described the proteome of the male and female gonad in fish. Early studies using 1D-SDS-PAGE and 2D gel electrophoresis in the ovary of zebrafish revealed that structural proteins (e.g. tubulins, beta-actin), binding proteins (e.g. heat shock proteins) and proteins with catalytic activity (e.g. enolases and transferases) were those most abundant and readily detectable in the female ovary [19]. Using 2D DIGE, MALDI-TOF, and nanoESI ITMS/MS, research conducted in the testis of Senegalese Sole characterized proteomic profiles during spermatogenesis [20]. The researchers demonstrated that proteins involved in the ubiquitin–proteasome pathway as well as those with a role in redox state are differentially expressed in wild and lab-reared sole, and this may be related to reproductive dysfunction (e.g. lower fertilization) observed in lab-reared fish. More recently, using Linear Trap Quadrupole (LTQ) mass spectrometers, a greater proportion of the teleost ovarian and testis proteome has been described. In zebrafish, Groh et al. [21] used 2D-LC-MS/MS (multidimensional protein identification technology, MudPit) to characterize global protein profiles of adult zebrafish gonad. The authors highlighted relevant proteins for gonad development and function and reported that proteins involved in cytokine and growth factor signalling systems, RNA- and DNA-interacting proteins, and proteins related to apoptosis were localized to the zebrafish testis and ovary. These studies provide the initial steps for characterizing the teleostean gonadal proteome. In this study, we used an LTQ Orbitrap Velos to describe the fathead minnow (FHM) (*P. promelas*) testis proteome. Moreover, we identified significantly enriched cell pathways represented by abundant FHM testis proteins. The FHM is a widely used freshwater species for ecotoxicology [22] and transcriptomics and proteomics methods for this species are becoming well developed. This study develops a proteomics framework for physiological and ecotoxicological studies focused on male FHM reproduction.

2. Methods

2.1. *Fathead minnow maintenance*

Reproductive adult male FHMs (2–3 years of age) were provided by FP Innovations (Pointe-Claire, QC, CAN). FHMs used in this study were housed at the Canadian Rivers Institute (Saint John, NB, Canada) in 400 L circular tanks. Both males and females were housed together and animals were maintained under flow-through conditions in dechlorinated Saint John city water at 25±2 °C, dissolved oxygen >90%, and under a 16 h light:8 h dark photoperiod. Fish were fed Corey Aqua Feed Optimum extruded fish pellets (Corey Feed Mills, Fredericton, NB, CAN). All animal use protocols were carried out ethically in accordance with Animal Care Committee protocol from the University of New Brunswick, Saint John (approval # 2011-3C-02).

2.2. *Histology*

For testes excision, three adult male FHMs were chosen based on the presence of significant banding coloration and the presence of nuptial tubercles, a prominent secondary sex characteristic for reproductive males. Following treatment with buffered ethyl-3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich), FHMs were sacrificed by severing the spinal cord. Testes were extracted, and after removing an explant for histology, tissues were snap frozen at −80 °C. The mean body weight was 7.0 g (SD=1.28) and the mean gonadosomatic index (GSI) was 1.44% (SD=0.060). GSI is a measure of gonad size relative to body weight and was calculated as [gonad wt/body wt]×100. The explants for histological examination were shipped to the diagnostic histology laboratory at the University of Prince Edward Island (UPEI) Atlantic Veterinary College (PEI, Canada). Sections were mounted on glass slides and stained with haematoxylin and eosin using standard protocols. Testes were examined using a Leica DM2500 bright field microscope at 10× magnification and images were captured using a Leica DF290 digital microscope camera (Fig. 1).

2.3. *Protein digestion, OFFGEL peptides electrophoresis, and LC-MS/MS*

For protein extraction, testes were homogenized in a phosphate-buffered saline solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH=7.4). Proteins were extracted in 5 times volume of 0.1 M ammonium acetate in 100% methanol. The pellets were washed twice with 0.1 M ammonium acetate in 100% methanol, once with 80% acetone and finally with 70% methanol. Protein pellets were resuspended with 1 M urea in 100 mM ammonium bicarbonate buffer and the protein concentration was assayed using the CBX kit (G-BioSciences, Maryland Heights, MO, USA). One hundred micrograms of protein were reduced with 10 mM DTT for 30 min, and then alkylated with 55 mM iodoacetamide for 45 min at room temperature in the dark. The samples were then digested with trypsin (Sigma, St. Louis, MO, USA) at a final enzyme/substrate ratio of 1:50 (w/w). The trypsin digest was incubated at 37 °C for 12 h. After digestion, the peptide mixture was dried down before de-salting using a Pierce C18 spin column (Thermo Scientific, Rockford, IL, USA). The peptide digest was resuspended in water for in solution pI-based electrophoresis using the 3100 OFFGEL fractionator (Agilent, Santa Clara, CA, USA). An aliquot of the digest was used to run directly by LC-MS/MS without fractionation in a preliminary experiment. These data were later combined with data from fractionation experiments to yield total proteins identified in the FHM testes.

The peptide digest was further separated into 12 fractions using 3–10 non linear IPG strips (GE-Healthcare, Piscataway, NJ, USA) at 8000 V for a total of 20,000 V. After separation, the fractions were transferred into tubes and dried down. All of the fractions and the aliquot were resuspended in 5% ACN/0.1% formic acid and analyzed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) coupled with a nanoLC