

IN SEARCH OF NOVEL FISH REPRODUCTIVE MARKERS: UTILISATION OF PROTEOMICS TO ELUCIDATE OOCYTE MATURATION REGULATORY MECHANISMS

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ABSTRACT

Using proteomics approach, this project seeks to isolate proteins involved in zebrafish oocyte development and maturation. Understanding the mechanisms regulated by these proteins would hopefully assist in enhancing the reproductive performance of aquaculture broodstock. We described here the background, objectives, general methodology and relevant results of our project. In summary, using proteomics approach combining 2D electrophoretic method and mass spectrometry, we described the identification of several proteins with previously unknown functions in ovarian biology.

OBJECTIVES

Reproductive performance of many farmed species showed inconsistencies when they are grown in captivity. An important aspect associated with this is the need to understand the physiological and biochemical aspects of reproduction-related events in the broodstock. In female fish, an array of pathways governed the development, maturation and eventually ovulation of the female oocytes. Understanding these pathways, followed by identification of major regulatory proteins controlling these pathways could possibly provide better avenues to control and manipulate the breeding of aquaculture fish species[1]. The immense significance of the zebrafish (*Danio rerio*) as a vertebrate model, due to its many advantageous innate qualities (e.g. optical clarity of the embryo, rapid embryonic development, high fecundity and fertility, ease of manipulation and maintenance), has been well recognised in the past two decades. Recent reviews have demonstrated that the zebrafish is an excellent model to study development, function, and regulation of the ovary [2].

Molecular tools investigating whole transcriptome or proteome of cells offer the possibility of mining for specific gene or protein markers related to specific stages of oocyte growth and development maturational stages. The term 'proteome' describes the protein complement of a genome. In essence, proteomics is therefore the study of protein properties (expression level, post-translational modification, interactions etc.) on a large scale to obtain a global, integrated view of disease processes, cellular processes and networks at the protein level [3]. We propose here the utilisation of

proteomics platform involving the separation capabilities of two-dimensional gel electrophoresis (2DE) with mass spectrometry to provide insights on the molecular mechanisms governing oocyte maturation in zebrafish for purpose of discovery of biomarkers useful for aquaculture reproduction.

METHODOLOGY

Late vitellogenic ovaries were sampled from 6 female zebrafish randomly chosen from the population tanks. Ovaries were transferred into microtubes followed by centrifugation at 13,200 rpm at 4°C for 20 minutes. The resulting pellet was then dissolved in rehydration buffer [8 M urea, 50 mM DTT, 4% CHAPS, 0.2% ampholyte 3/10, 0.0002% bromophenol blue and deionised distilled water].

Protein concentration was determined using RC DC protein assay kit. Analytical gels were prepared by passively rehydrating 17 cm pH 3-10 NL ReadyStrip IPG strips in 300 µL of rehydration buffer containing 60 µg of protein for 16 hours. Isoelectric focusing (IEF) was carried out using PROTEAN IEF cell at 250 V for 20 minutes, followed by 10,000 V (2.5 hours) and 10,000 V, 40,000 Vhr (4 hours). Following IEF separation, IPG strips were equilibrated with the first equilibration solution [6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol, 2% (w/v) DTT] for 15 minutes with gentle shaking. This is followed by another equilibration with a second equilibration solution [6 M urea, 0.05 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide] solution for 15 minutes with gentle shaking. Equilibrated strips were applied onto 15% SDS-PAGE gel for the second dimension separation using PROTEAN II XL vertical electrophoresis system at constant ampere of 16 A per gel for 30 minutes before increasing to 24 A per gel until the end of the electrophoresis run. Precision Plus Protein standard was used as molecular weight marker.

All gels were scanned using the GS-800 densitometer and protein spots were analysed using PDQuest version 7.3.1. Gels were analysed for spot detection, background subtraction and protein spot OD intensity quantification using the 3D imaging function in the software to eliminate artifact spots. For mass spectrometry analysis, spots of interest were excised from CBB gels using new scalpel blades and transferred to 200 µL microtubes. Following sample processing, the resulting sample peptide mixture was spotted onto the MALDI target plate, allowed to dry prior mass spectrometry analysis. Mass spectrometry was performed using the 4800 MALDI-TOF/TOF Analyzer using settings and parameters described earlier. MS-MS/MS data was interpreted using Data Explorer version 4.9. Peptide sequences were obtained by calculating the differences residue mass between the adjacent fragment ion peaks. MS/MS sequences were subjected to different protein database searching tools such as from NCBI, PROSITE, and Pfam to identify possible matches.

RESULTS

The approach used to extract total proteins, followed by treatment and isolation using 2D electrophoretic approach was able to produce good quality gels. These gels were analysed with spot-analysis software for spot integrity and quality. Subsequently, a total of 80 spots were selected and excised for mass spectrometry analysis.

Among proteins identified in this project with well-known roles in oocyte development are various isoforms of vitellogenins and zona pellucida glycoproteins[4]. More interestingly, we identified 10 proteins previously undescribed in ovarian biology with significant presence in zebrafish ovary (Table 1). These proteins should form the basis for future studies attempting to elucidate undiscovered pathways and mechanisms governing oocyte maturation [5].

Name	species
ATP synthase	<i>Danio rerio</i>
Calreticulin	<i>Danio rerio</i>
Aldehyde dehydrogenase	<i>Danio rerio</i>
Mitochondrial ATP synthase	<i>Danio rerio</i>
Voltage-dependent anion channel 1	<i>Danio rerio</i>
Prdx3 protein	<i>Danio rerio</i>
isovaleryl Coenzyme A dehydrogenase	<i>Danio rerio</i>
3-oxoacid CoA transferase 1a	<i>Danio rerio</i>
enoyl Coenzyme A hydratase	<i>Danio rerio</i>
Heat shock proteins	<i>Danio rerio</i>

Table 1. List of proteins with previously unknown roles in ovarian physiology isolated in this project

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