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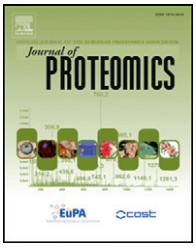

Kidney proteome responses in the teleost fish *Paralichthys olivaceus* indicate a putative immune response against *Streptococcus parauberis*

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ABSTRACT

The proteomic response to bacterial infection in a teleost fish (*Paralichthys olivaceus*) infected with *Streptococcus parauberis* was analyzed using label-free protein quantitation coupled with LC–MS^E tandem mass spectrometry. A total of 82 proteins from whole kidney, a major lymphoid organ in this fish, were found to be differentially expressed between healthy and diseased fish analyzed 6, 24, 72 and 120 h post-infection. Among the differentially expressed proteins, those involved in mediating immune responses (*e.g.*, heat shock proteins, cathepsins, goose-type lysozyme and complement components) were most significantly up-regulated by infection. In addition, cell division cycle 48 (CDC48) and calreticulin, which are associated with cellular recovery and glycoprotein synthesis, were up-regulated in the universal protein group, whereas the other proteins in that group were down-regulated. There was continuous activation of expression of immune-associated proteins during infection, but there was also loss of expression of proteins not involved in immune function. We expect that our findings regarding immune response at the protein level would offer new insight into the systemic response to bacterial infection of a major immune organ in teleost fish.

Abbreviations: LC, liquid chromatography; MS, mass spectrometry; UPLC, ultra performance liquid chromatography; CDC48, cell division cycle 48; EST, expressed sequence tag; iTRAQ, isobaric tag for relative and absolute quantitation; SILAC, stable isotope labeling by amino acids in cell culture; ICAT, isotope-code affinity tags; emPAI, exponentially modified protein abundance index; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; TSA, tryptone soya agar; TSB, tryptone soya broth; PBS, phosphate buffered saline; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ABC, ammonium bicarbonate; CAN, acetonitrile; DTT, dithiothreitol; IAA, iodoacetamide; PLGS, ProteinLynx Global Server; IPA, Ingenuity Pathway Analysis; PICR, Protein Identifier Cross-Reference; HSP, heat shock protein; LMP7, low molecular mass polypeptide 7; TLR, toll-like receptor; MHC, major histocompatibility complex; NAM, N-acetylmuramic acid; NAG, N-acetylglucosamine; IgM, immunoglobulin M; APCs, antigen presenting cells; ISG, interferon-stimulation gene 15; ER, endoplasmic reticulum; GLUT-1, glucose transporter 1 (GLUT-1); AAA ATPase, ATPases associated with various cellular activities.

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1. Introduction

The teleost fish is the primary vertebrate that helps in understanding the evolution of the immune system [1]. This lower vertebrate has an innate immune system and an apparent adaptive immune system, moreover, its eccentric immune composition supplies key clues to understand the evolution of the immune system of higher vertebrates. For instance, the kidney in fish plays a crucial part in immune responses. The major function of the kidney is to maintain body fluid homeostasis, the same as higher vertebrates, but additionally, it is a major lymphoid organ [2]. Indeed, the morphological, ultrastructural and cytochemical features of the reticulo-endothelial stroma in the head of the kidney in the gilthead sea bream *Sparus aurata* suggest that the function of the kidney in fish is in part analogous to the bone marrow in mammals [3]. Thus, a study of the primitive kidney of vertebrates as exemplified by fish is of particular importance to fully understand the immune system of all vertebrates.

A number of studies conducted on the olive flounder (*Paralichthys olivaceus*) as a model teleost have been reported. In particular, a genomic analysis of expressed sequence tag (EST) analysis of the liver and spleen of the olive flounder accelerated studies on this species [4]. Up to now, genomic engineering, including transcript analysis, has been used as the major analytic tool to screen for molecular responses in teleost fish, including the olive flounder. But while transcriptional analysis has been valuable in fish science and in comparative studies of phylogeny, it is unable to detect specific molecular interactions between cells that rely on functional signal delivery systems. In fact, mRNAs are defined as indirect or disposable messages that temporarily transmit information and have no other function, whereas proteins have relatively direct functions in biological processes [5]. Proteomics is a means of comprehensive interpretation, which could be used to describe more direct molecular responses than conventional genomics, which assess only the messenger [5]. Therefore, the performance of a concomitant proteomic and gene expression analyses would be expected to shed new light on the cellular responses in fish immunology.

Comparative quantitative proteomic analysis can entail the use of labeled or label-free protein quantitation with high throughput mass spectrometry (MS) [6]. Isobaric tag for relative and absolute quantitation (iTRAQ) [7], stable isotope labeling by amino acids in cell culture (SILAC) [8] and isotope-code affinity tags (ICAT) [9] are commonly employed for labeled quantitative proteomics. However the required attachment of isotope labels prior to the MS analysis is a complicated and time-consuming process, which makes the use of label-free quantitation desirable, when possible. Label-free protein quantitation is carried out through spectral counting or ion monitoring methods [6]. The exponentially modified protein abundance index (emPAI), as a spectral counting method, is one approach to approximate protein quantitation [10]. We previously applied emPAI calculation to determine the differences in hemolymph proteins between healthy and diseased tunics in ascidians suffering from soft tunic syndrome [11]. However, because of the weakness of approximate protein quantification, an improved proteomic quantitation method is used. Selected reaction monitoring (SRM) enables comparison of a single ion-intensity between experimental groups and can be expanded to cover multiple reaction monitoring (MRM) for multiple comparisons of ion-intensities between groups [6,12,13], this could offer more reliable results than emPAI analysis.

To our knowledge, there have been no proteomic analyses investigating the kinetics of the fish immune system. Therefore, we used a proteomics approach to perform a systemic biological analysis in the olive flounder. Detailed characterization of the complete response of an organism to an environmental hazard like an invasive microorganism is a challenging endeavor; however, we were able to form comprehensive inferences about host responses to a disease condition using bioinformatics analysis [14,15]. To accomplish this, the fish were experimentally infected with *Streptococcus parauberis*, which can cause massive mortality in olive flounder. The changes in the proteome between the healthy and diseased flounder were then examined using a label-free protein quantitation analysis with LC–MS^E tandem mass spectrometry. This report of the detailed proteome from the whole kidney of fish in the context of direct protein kinetics will contribute to an extrapolation of the responses of the immune system during bacterial infection.

2. Materials and methods

2.1. Experimental bacterial infection and tissue sampling

Healthy olive flounder fry 9±1 cm in length and weighing 7.5± 1.5 g (Supplementary Fig. S1) were obtained from a commercial fish farm in Namhae, Gyeongnam, Korea. The fish were transferred to the challenge facility in Gyeongsang National University. A total of 50 fish were kept in two 260 liter tanks at 26 °C for one week in filtered seawater. *S. parauberis* was isolated from diseased fish in Jeju, South Korea. The pathogen was identified through hemolytic and biochemical analyses, confirmed by PCR analysis [16], and was stored at −70 °C until use. Bacteria were cultured in tryptone soya agar (TSA; Oxoid Ltd., Basingstoke, UK) or tryptone soya broth (TSB; Oxoid Ltd.) supplemented with 2% NaCl at 27 °C. For experimental inoculation, the bacteria were cultured until the cell density reached an OD_{610} of 1.0, after which a 7×10^8 CFU suspension was prepared after washing with PBS. The fish were pooled and approximately 7×10^7 bacterial cells in PBS or the same volume of PBS solution (Control) were intraperitoneally inoculated into 25 fish each. The infected and control groups were separately maintained in a 260 liter tank at 26 °C. Five infected fish and five control fish were then sampled after 6, 24, 72 or 120 h. During the experimental period, the seawater in the tanks was partially replaced with fresh filtered seawater every day. The presence of the bacterium was tested from both groups 8 days post-infection but was detected from infected fish only (data not shown). Experimental infection and collection of organs were performed after anesthetization with Aqui-S® (New Zealand Ltd., Wellington, NZ). Whole kidneys were individually collected from each fish, after which each organ was divided into two sections, longitudinally. One half of each organ was stored at −70 °C with 100 μl