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Heat shock protein profiles on the protein and gene expression levels in olive flounder kidney infected with *Streptococcus parauberis*

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ABSTRACT

Heat shock proteins (HSPs) have been observed in cells exposed to a variety of stresses, including infectious pathogens. This study used a label-free, quantitative proteomic approach and transcriptional gene expression analysis to investigate infection-related HSP proteins and their encoding genes in whole kidneys from olive flounder (*Paralichthys olivaceus*). During *Streptococcus parauberis* infection in the flounder, the genes encoding Hsp10, Hsp40A4, Hsp40B6, Hsp40B11, Hsp60, Hsp70, glucose regulated protein 78 (Grp78), Hsp90α, Hsp90β and Grp94 were induced, and the protein levels of Hsp60, Hsp70, Hsp90α, Hsp90β and Grp94 were differentially regulated over time. Subsequent results also revealed that Hsp60, Hsp70, Hsp90α, Hsp90β and Grp94 appear to be the dominant and critical HSPs in olive flounder during bacterial infection. This is the first estimation of the differential involvement of HSPs in the immune response of olive flounder exposed to bacterial infection.

1. Introduction

As suggested by its name, heat shock protein (HSP) is induced to striking levels in response to environmental changes such as heat stress, where it functions to protect both prokaryotic and eukaryotic cells from environmental effects [1]. HSPs also play important roles in cellular responses to other noxious stimuli, including starvation, chemotherapeutic agents, inflammation, anoxia ischemia, and viral and bacterial infection [2–4]. Therefore, the term 'stress protein' has been proposed as an alternative nomenclature for HSP [3,5,6]. Induced HSPs, as sensor molecules to trigger a signal in response to cellular stress, has been profoundly studied in the mammalian immune system. Stewart and Young [4] reviewed HSPs involved in the innate immune system, including Hsp60, Hsp70, Hsp90 and Gp96, which can interact with immune cells via cell-surface receptor signaling molecules, such as CD91 for Hsp70, Hsp90 and Gp96 [7]; CD14, TLR2 and TLR4 for Hsp60 and Hsp70 [8–10]; and CD40 for Hsp70 [10].

A number of studies have described the roles of HSPs in the immune response of teleosts. For example, Hsp70 protein was elevated in coho salmon (*Oncorhynchus kisutch*) infected with *Renibacterium salmoninarum* [11], and the expression of its encoding gene was induced in juvenile rainbow trout (*Oncorhynchus mykiss*) infected with *Vibrio anguillarum* [12]. In contrast, Hsp70 gene expression was shown to be down-regulated in sea bream [*Rhabdosargus sarba* (Forsskal)] infected by *V. alginolyticus*, whereas the expression levels of the genes encoding Hsp60 and Hsp90 were unchanged [13]. In the olive flounder (*Paralichthys olivaceus*), Hsp40A4 gene expression in olive flounder embryonic cells (FEC) was markedly induced by viral antigens, such as UV-inactivated turbot (*Scophthalmus maximus* L.) rhabdovirus (SMRV), and Hsp40B6 and Hsp40B11 were weakly induced [14]. In another study, Northern blot analysis revealed the induction of Hsp70 in flounder embryos subjected to heat stress [15]. Although these studies demonstrated the participation of HPSs in the teleost immune system to help them cope with infectious diseases, they are rather limited and thus insufficient to thoroughly explain the mechanism of HSPs and its role in immune response.

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Therefore, this study was designed in order to extend the underlying knowledge on teleost HSPs through the analysis of HSP responses against Streptococcosis. The regulation of HSPs were examined in whole kidneys (a major lymphoid organ in fish) using both label-free quantitative proteomic analysis with a nano-UPLC-MS^E system and transcriptional gene expression analysis with quantitative real-time PCR (qRT-PCR). Our combination of proteomic and genomic approaches provided new complementary cellular-level insights into the HSP response.

2. Materials and methods

2.1. Fish and bacterial preparation

Healthy olive flounder fry, 9 ± 1 cm in length and weighing 7.5 ± 1.5 g, were obtained from a commercial fish farm in Namhae, Gyeongnam, South Korea. Fish were transferred to the challenge facility of Gyeongsang National University in Jinju and maintained at 26 °C for a week in filtered seawater while being fed commercial food designed for fry. The pathogen, *Streptococcus parauberis* (KCTC 11537 strain), was isolated from fish infected with streptococcal bacteria in Jeju. *S. parauberis* was identified by hemolytic analysis and biochemical assays, the identity was confirmed by PCR analysis, and the bacteria were stored at −70 °C until use [16]. For experiments, bacteria were cultured on 2% NaCl (w/v) Tryptone Soya Agar (TSA; Oxoid Ltd., Basingstoke, GBR) at 27 °C. A single colony was collected, cultured in 2% NaCl (w/v) Tryptone Soya Broth (TSB; Oxoid Ltd., Basingstoke, GBR) at 27 °C to an $OD_{610\ nm}$ of 1.0, washed with phosphate buffered saline (PBS; 3 mM KCl; 137 mM NaCl; 1.5 mM KH_2PO_4; and 8 mM Na_2HPO_4, final pH 7.4), and suspended to 7×10^8 CFU in PBS.

2.2. Experimental bacterial infection and tissue sampling

Olive flounder were inoculated intraperitoneally with either 7×10^7 bacterial cells in PBS or PBS alone (control). The experimental and control fish ($n = 5$ per group) were sampled at 6, 24, 72 and 120 h, respectively. Experimental infection and organ extraction were performed under anesthetized conditions using Aqui-S® (New Zealand Ltd., Wellington, NZL). Whole kidneys were used to investigate the response of HSPs to bacterial infection. The organs were collected individually from each fish, and each organ was divided into two longitudinal sections. One section was kept at −70 °C in 100 μl of RNA*later*® (Ambion, Austin, TX) for later use in cDNA synthesis; the other section was stored directly at −70 °C for LC-MS/MS analysis.

2.3. Sample preparation for LC-MS/MS analysis

The stored kidneys samples were individually homogenized with a T-10 basic homogenizer (IKA®, Staufen, DEU) and centrifuged at 16,000× g for 30 min. A protease inhibitor solution (Sigma, Rehovot, ISR) was used to minimize protein degradation. The supernatants were transferred to new microtubes (Protein LoBind Tube; Eppendorf, Hamburg, DEU) on ice, and the protein concentrations were examined using a BCA protein assay kit (Thermo Science, Rockford, IL). The proteins in the supernatants were precipitated with 10% trichloroacetic acid (TCA) on ice for 30 min followed by centrifugation at 16,000× g for 30 min. The supernatant was discarded, the pellet was washed with 1 ml of cold acetone, the sample was centrifuged at 16,000× g for 30 min, and the supernatant was discarded. This process was repeated twice. Thereafter, the protein pellet was allowed to dry, dissolved in 40 μl of Tris-buffered saline (TBS; 50 mM Tris–HCl and 150 mM NaCl, final pH 9.0), and then vortexed vigorously with 10 μl of 5× SDS sample buffer (0.2 M Tris, pH 6.8, 25% glycerol, 10% SDS, 5% 2-mercaptoethanol, 0.05% Bromophenol blue). The mixture was vortexed at RT for 30 min, incubated in boiling water for 5 min, and then 65 μg of protein from each sample was subjected to 12.5% (w/v) SDS-PAGE. After electrophoresis, the gel was stained with Coomassie G-250 (Bio-Safe TM; Bio-Rad, Carlsbad, CA), equally sliced into 10 segments, and completely destained by vortexing in equal volumes of 50 mM ammonium bicarbonate (ABC) and 50% acetonitrile (ACN) at RT. The gel strips were then reduced with 10 mM dithiothreitol (DTT) in 10 mM ABC at 56 °C for 1 h and alkylated with 55 mM iodoacetamide (IAA) in 100 mM ABC at RT for 45 min in the dark. The liquid was discarded, and the gels were treated with 500 μl of 100 mM ABC and dehydrated with 500 μl of 100% ACN. This process was repeated a second time, and the gels were vacuum dried. For digestion of the proteins, the dried gel fragments were rehydrated with 70 μl of trypsin solution (0.02 μg/μl; Promega, Madison, WI), incubated on ice for 40 min, and added 30 μl of 25 mM ABC. After incubation at 37 °C for 14 h, an additional 100 μl of 25 mM ABC was added, the samples were vortexed for 30 min, and the supernatants were transferred to new microtubes. Extraction solution [2.5% (v/v) formic acid in 25% (v/v) ACN] was added to the gel fragments, and the samples were vortexed at RT for 30 min. Finally, the supernatants were appropriately combined with the previous round of supernatants, and the resulting tryptic peptide mixture was kept at −70 °C until LC-MS/MS analysis.

2.4. Analysis by nano-UPLC-MS^E tandem mass spectrometry

Analysis of nano-UPLC-MS^E tandem mass spectrometry was performed according to the previously described method [17]. Briefly, the prepared peptide mixtures were desalted in a solid-phase Oasis HLB C18 microElution plate (Waters Corporation, Milford, MA) and used for LC-MS/MS analysis. The separations were performed on a 75 μm × 250 mm nano-ACQUITY UPLC 1.7 μm BEH300 C18 RP column and a 180 μm × 20 mm Symmetry C18 RP 5 μm enrichment column using a nano-ACQUITY Ultra Performance LC System (Waters Corporation). For long-term ionization stability, a recently developed spray tip was used as described [18]. Tryptic peptides (5 μl) were loaded onto the enrichment column with mobile phase A (3% acetonitrile and 0.1% formic acid in water). A flow rate of 300 μl/min was used, along with a step gradient that included the following: 3–40% mobile phase B (97% acetonitrile and 0.1% formic acid in water) over 95 min; 40–70% mobile phase B over 20 min; and then a sharp increase to 80% mobile phase B for 10 min. Sodium formate (1 μmol/min) was used to calibrate the TOF analyzer in the range of m/z 50–2,000, and [Glu^1]-fibrinopeptide (m/z 785.8426) at 600 nL/min was used for lock mass correction. During data acquisition, the collision energies of the low energy MS mode and the high energy mode (MS^E) were set to ramping energies of 4 eV and 15–40 eV, respectively. One cycle of MS and MS^E mode acquisition was performed every 3.2 s. In each cycle, the MS spectra were acquired for 1.5 s with a 0.1 s interscan delay (m/z 300–1990). The MS^E fragmentation (m/z 50–2000) data were collected in triplicate.

2.5. Protein identification and quantitative analysis

The continuous LC-MS^E data were processed and searched using the IDENTITY^E algorithm [19] from PLGS (Protein Lynx Global Server), version 2.3.3 with Expression version 2 (Waters Corporation). The data acquired from alternating the low- and elevated-energy modes in the LC-MS^E were automatically smoothed, background-subtracted, centered and deisotoped; the charge state was reduced prior to alignment with the precursor; and the fragmentation data