Doctoral Thesis

Gene Expression Analysis of Antimicrobial peptides in Ayu Stimulated with LPS.

(Summary)

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Summary

Fish supply from capture will be static or even decreased over the next 30 years. A growing percent of world aquatic production derives from aquaculture, whose importance increased dramatically as a result of the overfishing and increase the demand for sea food. In fact, aquaculture production has increased from 9% of the fisheries resources in 1980 to actually current 43%. It is thought that production will need to double in the next 25 years according to the FAO. Fish is free-living organism exposed to stress problems such as diseases and deterioration of environmental conditions often results in economic losses. Most of this causative agents in fish ponds is bacterial in source. And even with good management practice the losses may be up to 10% which equates to nearly $ 5 billion per year.

The most ancient and efficient line for defenses the fish against this microbes is the innate immune responses. They respond in short time scale and efficient manner with one of its alarm arms represented in our study by the antimicrobial peptides stimulated with lipopolysaccharides (LPSs) a pathological component. We aimed to set up analysis profile

of the ayu antimicrobial peptides stimulated with LPS and clear out the association of LPS recognition in various ayu groups. This practiced in (1), analysis of cathelicidin gene expression from liver tissues which stimulated with LPS different doses in different life stages. (2), analysis of hepcidin encoded by *LEAP-1* gene expression from liver tissues which stimulated with LPS different doses in different life stages. (3), analysis of cathelicidin gene expression from various tissues at portal of pathogens entrance (mucosal surfaces).

The second chapter included the first item, we have studied the relative transcriptional level of the cathelicidin gene in vivo stimulated with LPS of different doses 1 μg, 10 μg and 100 μg LPS/fish. LPS was injected intraperitoneal to ayu at different ages; young immature, mature and sexual mature adults of total 195 ayu purchased from Takahashigawa Fisheries Cooperative Association (Okayama, Japan). During the season from April 1st until the end of October. They were kept in 500 L plastic water tanks at 20 ºC ± 2, and fed regularly three times a day with commercial pelleted ayu diet. Liver tissues were collected three times. First time (group 1), in the mid-April, internal examination showed complete absence of the sexual organs. Their average body weight were 8-9 g and average body length were 5-6 cm. The second time (group 2), in the end May, mature fish their average body weight were 15-25 g and average body length were 8-10 cm. The third sample collection (group 3), in the mid-October, they were sexual mature adults, internal examination confirmed full raipned sexual organs in both sex just before the spawning. Their average body weight were 40-60 g and average body length were 15-20 cm. The relative expression level of the cathelicidin was measured using semi-quantitative RT-PCR. The expression level estimated and normalized to *actb* gene expression level with Image-

J software. All the data exposed to statistical analysis with ANOVA and student t-test. The results showed a direct association between the cathelicidin mRNA expression and the LPS used for the induction. Young fish showed significant up regulation in a time-, dose-dependent manner while mature and sexual mature fish showed non-significant change. We concluded that young fish is relay mainly on its innate immune response than adults.

The third chapter included the second item, we have studied the relative transcriptional level of the hepcidin encoded by *LEAP-1* gene using the same cDNAs sample used to the analysis of cathelicidin mRNA expression. The relative expression level of the hepcidin was measured using semi quantitative RT-PCR. The expression level were normalized to *actb* gene expression level with Image-J software. All the data exposed to statistical analysis with ANOVA and student t-test. The results showed a direct association between the hepcidin mRNA expression and the LPS used for the induction. Young, mature and sexual mature adults showed up regulation mainly in age-dependent manner. As the young and mature fish showed up regulation in a dose and time dependent manner. In the other hand, sexual matured fish showed significant down regulation. We could concluded from the first and second experiment that the hepcidin may be involved more in the ayu defenses against stressors such as pathogens.

In the fourth chapter, the relative quantitation of cathelicidin expression level was analyzed for 25 young ayu in the mid-April. 20 fish were divided to two groups injected intraperitoneal with PBS or LPS 1µg/fish. Tissues sample were collected from various organs (liver, gill, skin and intestine) at a time schsdule of 0 hr (5 control fish), 6 hr and 24 hr post injection. The expression of cathelicidin mRNA were analyzed in the three various tissues (gill, skin and intestine) with RT-qPCR. And normalized to the *actb* gene expression

level. ΔC_t and $\Delta\Delta C_t$ value were determined with the automatic setting of the programmed system software. All data analyzed in EXCEL 2013 software (ver. 15.0.4420.1017). The data depend on ΔC_t value subjected to ANOVA followed by t-test is representing the mean ±SD (n=5). *; $P \leq 0.05$ consider significant. Data depend on $\Delta\Delta C_t$ value is representing the mean (n=5). It considered ± 2 fold of change in cathelicidin mRNA expression levels to be significant. The constitutive cathelicidin expression from the mucosal surfaces were higher than that of liver tissues. While the induced expression of cathelicidin with LPS showed only significant decrease in gill and skin at 24 hr after stimulation. The results clear out that the constitutive and LPS inducible expression of the cathelicidin is under developmental control and the recognition of LPS may be tissue-specific although the mechanism of LPS recognition still unclear and also how this mechanisms affect in organs set distal to site of the immunostimulant administration (intraperitoneal in our study).

In conclusion the ayu antimicrobial peptides seems to play important role in ayu immune defenses against pathogens although it is age, time, dose and tissue-specific dependent production. Also, to how much this antimicrobial peptides involved in ayu defenses may depend on their families.

Further investigation is required for analysis both hepcidin isoforms and production of monoclonal antibodies to clear the post translation regulation of ayu antimicrobial peptides.