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Introduction

The immune system of animals consists of both acquired and innate immune systems. Fishes have a target-specific acquired immune system that uses antibodies, but it is less developed rather than that of mammals. To compensate for this, fish's innate immune system—which acts nonspecifically against pathogens, is essential. The fish innate immune system is composed of various defense substances, one of which is L-amino acid oxidase (LAO). Fish LAO was identified as an antibacterial protein from the body surface mucus and blood in black rockfish Sebastes schlegelii, Atlantic cod Gadus morhua, red-spotted grouper Epinephelus akaara, et cetera. This enzyme catalyzes the oxidative deamination of L-amino acids and converts them to α-keto acids, ammonia, and hydrogen peroxide. Hydrogen peroxide is known to act as an antimicrobial agent in fish because of its potent oxidative bactericidal effect. In addition, the LAO genes are widely identified in fish species and are induced by infection with pathogenic bacteria in Atlantic cod G. morhua, Atlantic salmon Salmo salar, hybrid tilapia Oreochromis spp., and zebrafish Danio rerio. It suggests that LAO is also an immunologically functional molecule. On the other hand, the results of the preliminary studies have revealed that LAO enzymatic activity was detected in limited fish species despite the broad range of LAO gene distribution. In this study, I try to clarify how LAO works in the “less-LAO” fish species. Based on the preliminary study, zebrafish are one of the less-LAO fish, and I used the species for the studies below: First, I identified which tissues contain the LAO gene and function as an enzyme. Second, I tried to detect the LAO protein in each tissue. Third, I developed an ultra-sensitive assay to confirm LAO enzyme activity.

Methods

LAO gene expression levels in each tissue (skin, gill, kidney, liver, spleen, intestine and muscle) of zebrafish were measured. Total RNA was extracted from each zebrafish tissue using ISOGEN (Nippon Gene Corporation, Tokyo, Japan). The cDNA was synthesized using the total RNA and reverse transcriptase, and PCR was

performed using zebrafish LAO gene-specific primers that were designed based on the database information. PCR was also performed with primers for the housekeeping gene rpl13a as a positive control. LAO protein in each zebrafish tissue extract was confirmed by Western blotting with a custom-made zebrafish LAO-specific antibody. The tissue extracts were prepared from skin, gill, kidney, liver, spleen, intestine, and muscle using beads tissue disruptor. The supernatants of the homogenates were used as the extracts. The extracts were applied onto SDS-PAGE and blotted to PVDF membrane; subsequently, LAO was reacted with the anti-LAO antibody and its secondary antibody. The positive bands were visualized using diaminobenzidine as a chromophore.

For future work, the fluorescent HPLC-based high-sensitive LAO assay was developed to detect feeble LAO activity in the samples. The principle of this method was based on the coumarin-Fenton reaction system—the hydroxy radical generated from hydrogen peroxide by the Fenton reaction converted non-fluorescent coumarin to fluorescent 7-hydroxyl coumarin. To compare the sensitivity of both the conventional colorimetric ortho-phenylenediamine/peroxidase (OPD-POD) method and the HPLC method, I used the dilution series of the grouper Epinephelus akaara serum as known LAO sample with/without substrate amino acid.

Results and Discussions

The results of PCR showed that rpl13a was expressed in all tissues, whereas LAO gene was detected in all tissues, but the expression level was varied. Especially skin and gills were detected from all test specimens. It is reasonable to understand about the host-defense mechanism by LAO, to avoid invading the pathogenic bacteria from these tissues. In contrast, Western blotting results suggested that LAO protein was not detected in any tissue extracts, because LAO protein amount could be very less in healthy zebrafish.

A newly developed fluorescent HPLC-based LAO assay successfully detected 0.51 μM of the LAO-born hydrogen peroxide in 1/3,000 dilution of E. akaara serum. In contrast, the conventional method could minimally detect 69.4 μM in 1/300 serum. These results suggested that the fluorescent HPLC method may have a 100 times higher sensitivity of hydrogen peroxide detection than the conventional method. However, the enzyme amount was only ten times higher than that. I will try to optimize the more low-concentration LAO enzymatic activity. In addition, the LAO activities in zebrafish tissues have to be measured using the Fluorescent HPLC-based LAO activity measurement. The conditions for induction of the LAO gene are also examined to confirm the expected role of LAO as a biological defense substance in zebrafish. In addition, the role of LAO in less-LAO fishes will be investigated, not only in the direct bactericidal action of the produced hydrogen peroxide but also in its involvement in redox signal transduction related to immunity.

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