組織化学的手法による魚類抗菌タンパク質の局在について

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Hazuki YAMAMOTO, Yoichiro KITANI: Histological analysis of the antibacterial protein in fish

Introduction and purpose

All lives have complexed host-defense mechanisms to avoid the diseases – it called the immune system. The immune system consists of two types of systems: innate immunity (primitive and nonspecific) and adaptive immunity (sophisticated and specific). In fish, innate immunity is essential because of the poor adaptive immunity traceable to the less complexity of the lymphoid organs. Several substances were founded as the innate immune molecules from fishes, such as lysozymes, lectins, antimicrobial peptides, *et cetera*. However, unidentified innate immune molecules still exist. Previously, we identified an L-amino acid oxidase (LAO) as an antibacterial protein from the skin mucus and serum of the marine teleost (Kitani et al., 2007). The LAO is an amino acid metabolism enzyme and generates ammonia, alpha-keto acid and hydrogen peroxide. The LAO acts on various bioactivities such as apoptosis, antivirus activity and antibacterial activity via resulted hydrogen peroxide. However, few studies refer to the immunological functions of LAO in fish. In the case of Atlantic cod and Atlantic salmon, LAO gene expression was upregulated by the pathogen exposure and it suggested that LAO relates to infection control (Kitani et al., 2015, 2019). Recently, the novel LAO was isolated from the serum of the red-spotted grouper *Epinephelus akaara* in our lab (Osaka & Kitani, 2021). The grouper LAO was 450 kDa (67 kDa subunits) and could react with L-Methionine, L-Phenylalanine and L-Tryptophan. The grouper LAO gene was not altered by the pathogen injection, different from other fishes mentioned above. Interestingly, the grouper LAO gene was strongly induced in the head kidney one day after blood loss. This result suggested that grouper LAO is necessary to avoid pathogen invasion via a wound.

However, the detail of the LAO regulation system is still unclear. Identification of the LAO producing tissue and/or cell type could help understanding the LAO production mechanism in the grouper. In this study, we tried to clarify the intra-tissue and inter-tissue localization of the grouper LAO protein using immunochemical/immunohistochemical methods with an anti-grouper LAO antibody.

Materials and methods

To recognize LAO protein-containing tissues, the tissue extracts were prepared from the healthy red-spotted grouper tissues as follows; skin, gill, muscle, stomach, intestine, liver, spleen, head kidney, trunk kidney, brain and heart. Each tissue was homogenized with the phosphate buffered saline (PBS, pH 7.0) using a reciprocal beads disruptor. The homogenates were centrifuged (18,000 × *g* for 15 min at 4 ℃) and the supernatants were used as the tissue extracts. The serum sample was diluted with PBS. These extracts were applied on SDS-PAGE (2.5 μg/lane) and electrophoretically blotted onto polyvinylidene difluoride membrane. The membrane was treated with a blocking solution; subsequently, anti-grouper LAO antibody and horseradish peroxidase-

conjugated secondary antibody. The grouper LAO cross-reactive protein was visualized by 3-amino-9-ethylcarbazol. In addition, the LAO activity of these tissue extracts was measured by the peroxidase/*o*-phenylenediamine method.

To observe the localization of the LAO protein, immunohistochemistry of the head kidney was tried as a first step. The head kidney was dissected and embedded into a frozen-sectioning compound without fixation and quickly frozen by dry ice-hexane coolant. The frozen block was sliced a thickness of 5 μm using a cryostat and mounted onto the slide glass. The slices were treated with a blocking solution—subsequently, anti-grouper LAO antibody and horseradish peroxidase-conjugated secondary antibody. The grouper LAO cross-reactive protein was visualized with 3,3'-diaminobenzidine substrate solution and observed by a light microscope.

Results and Discussion

LAO activity measurement showed that the strongest activity was detected in serum, followed by intestine, skin, gill, head kidney and heart (n=3). The LAO cross-reactive protein (70 kDa) was detected dominantly in serum, following skin, gill, head kidney, heart and spleen. Similar results were observed in the other grouper (n=3). This reaction disappeared by the neutralization of the anti-grouper LAO antibody with the purified grouper LAO. Both experiments reflected that the LAO activity detected from these tissues was caused by the grouper LAO cross-reactive protein except the intestine. LAO activity in the grouper intestine might be caused by unknown LAO that could not detect by anti-grouper LAO antibody.

In the head kidney, anti-grouper LAO antibody was localized at blood vessel (BV), the periphery of renal tubes and vascular sinusoids in collecting tubules and hematopoietic tissue. The positive signal was also detected at adenoid tissue (glandular, sparse, lymphoid tissue-like). These signals disappeared by the neutralization of the anti-grouper LAO antibody with the purified grouper LAO. Similar reactions were found in the other specimen (n=4). In addition, the positive signals were observed in skin and gills. These results suggested that the grouper LAO may act as a host defense molecule to protect whole body protection from invaders.

Reference

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