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Yuto OSAKA and Yoichiro KITANI: Purification and characterization of the antibacterial L-amino acid oxidase in the Red-spotted grouper *Epinephelus akaara* serum

【Background】

Fish body surface has a barrier which protects their body from attacks of pathogens. Recently, we discovered the L-amino acid oxidase (LAO) as a host-defense molecule in fish skin and blood (Kitani et al., 2007, 2010). This molecule elaborates the hydrogen peroxide by oxidization of the L-amino acid substrate. (Fig. 1) The fish defense mechanism that mediates LAO and hydrogen peroxide would be an efficient host-defense system; nevertheless, it is still unclear. In this study, to understand the physiological and biochemical functions of LAO, we try to search the diversities of fish LAO and clarify its structure.

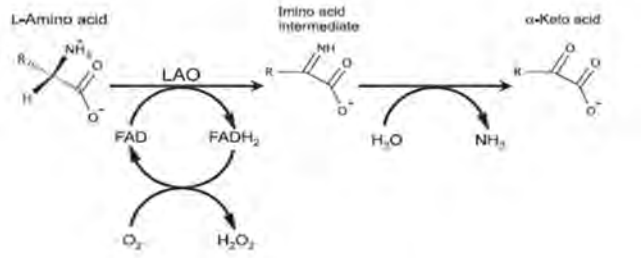

Fig.1. Reaction model of LAO

【Purpose】

The aim of this study is to search fish species which is not yet known to have LAO in their body and to elucidate substrate specificity and structure of the LAO of that fish species.

【Method】

First, inter-fish species screening of serum LAO activity was carried out. The specimens were collected from Tsukumo bay, Noto Peninsula, Ishikawa Japan, and serum samples were prepared. In this study, proteinogenic 20 kinds of L-amino acids were used as LAO substrate. This LAO activity assay was carried out following Figure 2. LAO catalyzes the L-amino acids and subsequently generate hydrogen peroxide. To detect the hydrogen peroxide, peroxidase (POD) and *o*-phenylenediamine (OPD) were added. OPD is oxidized and colored with the hydrogen peroxide by POD. This is the principle of LAO activity assay. Second, antibacterial activity of serum of those fishes was performed by agar diffusion and micro dilution assay methods. Third, isolation of serum LAO was tried by a combination of HighQ anion exchange chromatography, CHT hydroxyapatite HPLC and Superdex S-200 gel filtration HPLC. Elution of LAO was monitored by absorbance at 280 nm and LAO activity. The purity of serum LAO was judged by SDS-PAGE. Final, protein sequences of the purified serum LAO were determined. For N-terminal sequencing, the protein was transferred onto PVDF membrane and cut the target protein. For internal amino acid sequencing, purified LAO was digested by the lysyl endopeptidase. The digests were subjected to reversed phase HPLC to

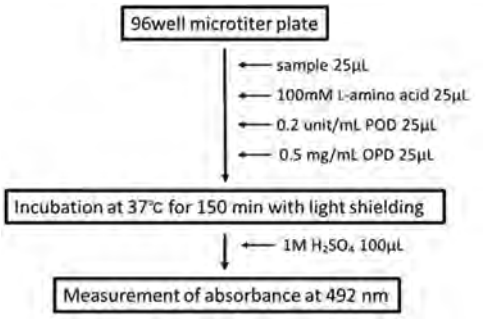

Fig. 2. flowchart of LAO activity assay

collect peptide fragments. Then, amino acid sequencing was analyzed by Edman degradation based protein sequencer.

【Result】

As a result of inter-fish species screening of serum LAO activity, the serum of Red-spotted grouper *Epinephelus akaara* (Kijihata) showed the activity (Fig. 3). *E. akaara* is one of the marine fish species which captured in Coast of Japan Sea as precious fish. *E. akaara* serum catalyzed a broad range of L-amino acid substrates such as L-histidine, L-methionine, L-phenylalanine, and L-tryptophan. *E. akaara* serum showed antibacterial activity against *Aeromonas salmonicida* and *Vibrio anguillarum* which are well known marine pathogenic bacteria. This activity was disappeared by the adding of catalase. Those results suggested that the antibacterial activity of *E. akaara* serum is caused by hydrogen peroxide. Purification of the *E. akaara* serum LAO was succeeded by the three-step chromatography described above. As a result of purification, *E. akaara* serum LAO is an acidic protein with molecular mass of 440 kDa and 70 kDa that estimated by gel filtration HPLC and SDS-PAGE, respectively. This suggests that *E. akaara* serum LAO is a multimeric enzyme *in vivo*. The N-terminal amino acid sequence of *E. akaara* serum LAO determined to DDITEVPDD and two of internal peptide sequences determined to NEEEGWYVELGAM and YDVWPSEK, respectively. Those sequences were highly similar to LAO of other fishes. In conclusion, *E. akaara* serum contains antibacterial LAO. This molecule may play a role as a host-defense molecule against invasion and infection of bacteria from the wound.

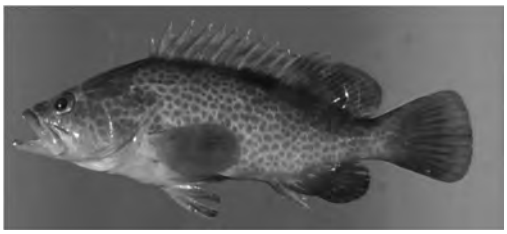

Fig. 3. *Epinephelus akaara*

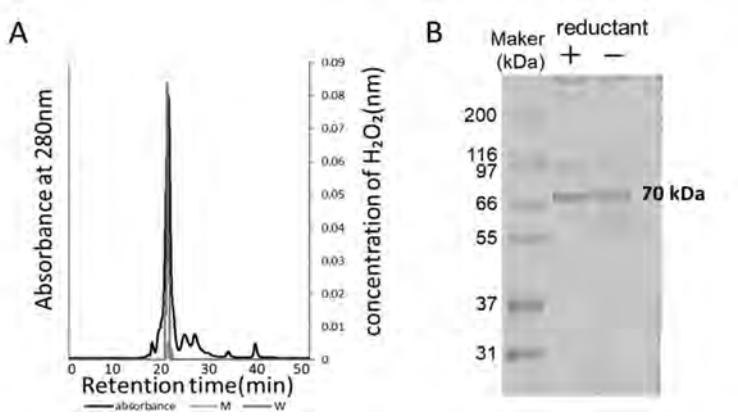

Fig. 4. Purified *E. akaara* serum LAO
A; chromatogram of gel filtration HPLC, B; SDS-PAGE of LAO.

【References】

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