報告番号	※　　第　　号

主　論　文　の　要　旨

論文題目　Functional studies of the egg cortical alveolus proteases on fertilization of medaka
(メダカの受精における卵表層胞局在プロテアーゼの機能解明)

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論　文　内　容　の　要　旨

In most organisms, fertilization is a starting point of embryonic development, in which dormant eggs are activated by sperm to trigger rapid changes for early development and ontogenesis. In fish eggs, the egg plasma membrane is surrounded by chorion, a thick extracellular proteinaceous layer. Under the egg plasma membrane, there exist secretory vesicles called cortical alveoli (CA) in the cortical cytoplasm. Immediately after fertilization, the CA fuse with the plasma membrane to undergo exocytosis and discharge of their contents into the perivitelline space (PVS), a space between the plasma membrane and the chorion. Subsequent enlargement of the PVS and the concomitant elevation of the chorion are accompanied by a chorion hardening in which the soft chorion changes to the hard chorion. A series of these events are called the cortical reaction. One of the prominent events is a proteolytic processing of the CA component, hyosophorin. Hyosophorin is a major sialic acid-rich glycoprotein, and is present as a high-molecular-weight form (H-hyosophorin), consisting of tandem-repeat structure of the glycononapeptide. During the cortical reaction, H-hyosophorin undergoes proteolytic depolymerization to the least repeating unit, L-hyosophorin. An enzyme responsible for the depolymerization of H- to L-hyosophorin was named as hyosophorinase, which has remained unidentified yet. Another drastic event during the cortical reaction is chorion hardening. It has been shown that a CA-localized protease, alveolin, is involved in chorion hardening in medaka. Alveolin is known to cleave the ZPB, a major chorion component; however, how alveolin facilitates the chorion hardening in vivo has remained unknown. It has also remained unknown if alveolin is also involved in the depolymerization of hyosophorin. Thus, the objective of this study is to understand how CA-localized proteases are involved in hyosophorin depolymerization and chorion hardening during cortical reaction, and to

attain this, focusing on alveolin and its related proteases in medaka eggs, the following experiments were carried out: (1) Search for CA-localized proteases and generation of their deficient medaka; (2) Functional analysis of alveolin *in vivo* using alveolin-deficient medaka; (3) Identification of the hyosophorinase; (4) Analysis of multiple genes for the major CA-localized glycoprotein hyosophorin.

(1) Search for CA-localized proteases and generation of their deficient medaka (Chapter 2): Although alveolin was already identified to be involved in chorion hardening in medaka, it shared the common properties with presumptive hyosophorinase: (a) CA- and PVS-localization before and after fertilization, respectively; (b) increased activity at fertilization; (c) specific cleavage of the peptide bond before Asp residue. Accordingly, gene databases were searched for alveolin-like proteases. As a result, a zinc metalloproteinase Nas-4 showing 45% identity in the amino acid sequence was found. Then, the *alveolin*-deficient medaka (*Alv*-KO) and the *Nas-4*-deficient medaka (*Nas-4*-KO) were generated using the CRISPR/Cas 9 gene editing technology to evaluate effects on the depolymerization of hyosophorin at the organism level. In both *Alv*-KO and *Nas-4*-KO medaka, the sperm could fertilize the egg, and the embryo normally developed, grown up, and produced offspring.

(2) Functional analysis of alveolin *in vivo* using *alveolin*-deficient medaka (Chapter 3): Since the most prominent feature of *Alv*-KO embryos was that chorion was mechanically fragile, properties of the chorion from *Alv*-KO and wild-type medaka (WT) were analyzed by the measurement of mechanical toughness, light and transmission electron microscope (TEM) observations, the measurement of permeability, and biochemical analysis of chorionic components. First, the chorion diameter of *Alv*-KO was larger than that of WT, due to the expansion of PVS. Second, the thickness of chorion was thinner in *Alv*-KO than WT, although the number of multiple layers remained unchanged. Interestingly, the TEM observation showed that the chorion structure of *Alv*-KO was collapsed at the outer part of the inner layers. In addition, the soft chorion was permeable to at least 10 kDa FITC-dextran, in contrast with the WT chorion. Moreover, although the crosslink between ZPB and ZPC happened in the *Alv*-KO like WT, crosslinking process was extremely delayed in *Alv*-KO. However, the delayed hardening process was accelerated in the balanced salt solution (BSS), compared with the case in water, although its acceleration was far slow than the WT case. Notably, alveolin processed not only ZPB, but also the chorion-localized transglutaminase at fertilization, which was a new finding.

(3) Identification of the hyosophorinase (Chapter 4): To analyze if alveolin and Nas-4 are involved in the depolymerization of H-hyosophorin, the H- and L-hyosophorin fractions in the fertilized embryos were quantified for *Alv*-KO and *Nas-4*-KO. In *Nas-4*-KO, H-hyosophorin was not depolymerized, while in *Alv*-KO, it was still depolymerized, although its amount was decreased. Interestingly, the decreased H-hyosophorin in *Alv*-KO

embryos was found in the culture medium, because the Alv-KO chorion was leaky to hyosophorin. These results indicate that Nas-4 is the hyosophorinase that depolymerizes H-hyosophorin during cortical reaction.

(4) Analysis of structural features of hyosophorin family in fish (Chapter 5): The recently updated databases were surveyed to find the hyosophorin gene not only in medaka and rainbow trout, but also in carp, northern pike, and tilapia. It has been shown that hyosophorin has multiple genes, each of which consists of three regions, including N-region, a major R-region consisting of tandem-repeats of completely the same sequences, and C-region. In rainbow trout, 54 hyosophorin genes found in this study contained largely the same gene structure as those reported previously. On the other hand, 18 genes were detected in medaka and half of them contained significant mutations in the R-region, which does not match the common concept of the hyosophorin gene. These results suggest that at least two different features in terms of conservation of the R-domain structure in evolution, which might be interesting to understand the significance of the CA contents and conditions of fertilization in various fish.

In conclusion, this study first demonstrates that Nas-4 is the hyosophorinase responsible for the proteolytic depolymerization of hyosophorin during cortical reaction in medaka. It also demonstrates that alveolin facilitates rapid chorion hardening via proteolytic cleavage of the ZPB and a processing transglutaminase, followed by the ZPB-ZPC crosslink. Thus, this study for the first time clarified the significance of cortical alveolus proteases at fish fertilization at the organism level. The data obtained in this study have not only advanced the knowledge of biology of fertilization, but also have enhanced the usefulness of medaka as a model animal for biological and pharmacological applications.