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An oocyte-specific astacin family protease, alveolin, is released from cortical granules to trigger egg envelope hardening during fertilization in medaka (*Oryzias latipes*)

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ABSTRACT

It has long been hypothesized that in fishes the contents of cortical granules are involved in the hardening of egg envelope following fertilization. We previously purified the egg envelope hardening initiation factor from the exudates released from activated medaka (*Oryzias latipes*) eggs and tentatively termed this protein alveolin. Alveolin is a member of the astacin metalloprotease family and was proposed to be a protease which hydrolyzes ZPB at one restricted position to allow starting cross-linking with ZPC. Here, we investigated the complete pathway from biosynthesis and accumulation to secretion of alveolin. A single alveolin transcript was detected only in ovarian preparations, confirming the specific expression of alveolin in the ovary. In situ hybridization indicated that the alveolin mRNA is already expressed in the very early previtellogenic oocytes. However, immunocytochemical studies revealed that the appearance of alveolin protein was delayed until the beginning of the vitellogenic stage. The cortical granules isolated from unfertilized eggs contained a high molecular weight form of glycosylated alveolin with a 50 kDa relative molecular mass. Hypotonic treatment burst isolated granules in vitro and transformed alveolin to a 21.5 kDa form, which is the same size as that of natural alveolin released from eggs upon fertilization. This transformation was inhibited in the presence of leupeptin and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), suggesting that a serine protease is involved in alveolin activation upon fertilization. Furthermore, the phylogenetic relationship of alveolin with other vertebrate astacin family members was analyzed. The result shows that alveolin and its teleostean homologs make a new group which is separate from either the hatching enzyme, meprin and BMP1/tolloid groups.

Introduction

In most animals, the egg is enveloped by an extracellular coat. The coat of vertebrate eggs has been termed by different names in different classes: zona pellucida in mammals; perivitelline layer in birds; vitelline envelope in amphibians; and chorion in fishes, and is generally referred to as the egg envelope (Dumont and Brummett, 1985). Despite the difference in names, the egg envelope of all vertebrate classes is mainly constructed of ZP glycoproteins, which have highly conserved ZP-domain and assemble to form filamentous supermolecular structures (Jovine et al., 2002). The ZP proteins have been classified by gene phylogenetic analysis into six subfamilies: ZPA/ZP2, ZPB/ZP4, ZPC/ZP3, ZP1, ZPAX, and ZPD (Goudet et al., 2008). In the teleost fish medaka, two types of ZPB (choriogenin H and choriogenin Hminor) and one ZPC (choriogenin L) are the major egg envelope components (Sugiyama and Iuchi, 2000).

Progressive biochemical and structural changes in the egg envelope occur during fertilization. From these modifications, the egg envelope loses its ability for binding and penetration by additional sperm and becomes resistant to mechanical, chemical and enzymatic disruption. These modifications play important roles in the

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prevention of polyspermy and in protecting the developing embryo from mechanical shock and bacterial infection. The contents of the cortical granules, which are exocytozed into the perivitelline space in response to sperm stimulation, are involved in transforming the egg envelope. The mechanisms underlying these modifications by cortical granule contents have been extensively investigated in sea urchin, frogs and mammals (reviewed by Wessel et al. (2001)). As one of these modifications, it has been reported that ZPA/ZP2 is specifically cleaved by a protease, released from cortical granules, and this cleavage lead to conformational changes in the supermolecular structure of the egg envelope proteins in mammals (Drobnis et al., 1988) and in frogs (Bakos et al., 1990). Recently, an oocyte-specific astacin-like proteae, ovastacin, was demonstrated as the cortical granule-derived ZP2 protease in mice (Burkart et al., 2012).

In the case of fish, the increase in resilience of the egg envelope after fertilization is remarkable. For example, unfertilized eggs of the brown trout (*Salmo trutta*) and the Atlantic salmon (*Salmo salar*) can resist a pressure of 160–280 g, whereas those 3–7 days after fertilization can resist a pressure as high as 2500–3500 g (Zotin, 1958), resulting from the hardening of their egg envelope. Although this hardening of the egg envelope also had been thought to be caused by cortical granules (also referred to as 'cortical alveoli' in fish) materials, definitive proof is lacking (reviewed by Hart, 1990; Iwamatsu, 2000). The molecular mechanism promoting hardening is now known to involve intermolecular cross-linking between egg envelope proteins catalyzed by transglutaminase (TGase) via the ε-(γ-glutamyl)lysine-isopeptide bonds (Hagenmaier et al., 1976; Oppen-Berntsen et al., 1990), resulting in toughening of the egg envelope. Thus, the 'engine' of the egg envelope hardening process is TGase. TGase has been shown to be embedded in the egg envelope in various fishes, such as rainbow trout (Ha and Iuchi, 1998), medaka (Iuchi et al., 1995) and carp (Kudo et al., 2000; Chang et al., 2002). However, this embedded TGase does not show any activity until the cortical reaction occurs (Iuchi et al., 1995). This has led to the idea that some substance discharged from eggs during the cortical reaction might be connected with the TGase action. Previously, we established an in vitro system to assess hardening inducing activity using isolated egg envelope and egg exudates, obtained from eggs by activation after removal of egg envelope layers (Iwamatsu et al., 1995). Subsequently, we identified the hardening inducing factor, alveolin, after purification from the exudates (Shibata et al., 2000). Alveolin is a member of the astacin metalloprotease family that was proposed to be a protease which hydrolyzes ZPB at one restricted position to start cross-linking with ZPC. Thus, alveolin, which is derived from the activated egg, acts as 'trigger' of the egg envelope hardening upon fertilization. It is thought that the egg envelope hardening in fish is carried out in two steps: (i) initiation by cortical granule derived protease, alveolin, and (ii) polymerization of egg envelope proteins with cross-linking formation catalyzed by TGase.

The aim of the present study is to determine the relationship between cortical granules and alveolin. Moreover, to clarify the initiation process of egg envelope hardening in fishes, we investigated the synthesis, localization and secretion of alveolin. Here, we provide the first direct evidence that synthesized alveolin accumulates into cortical granules as a proenzyme form and is released as an active form at the time of cortical granule breakdown (exocytosis).

Materials and methods

Preparation of oocytes and eggs

Medaka, *Oryzias latipes* (orange-red variety), were purchased from a fish farm (Yamako Fish Farm, Yatomi, Japan) and maintained under artificial reproductive conditions (26–28 °C, 14 h light/10 h dark cycle) for at least 2 weeks before the start of experiments. For the studies of early developmental stages, Qurt strain was used as described elsewhere (Shibata et al., 2010). The ovaries and oocytes were obtained according to the methods described by Iwamatsu et al. (1976) and kept in medaka saline (111.2 mM NaCl, 5.4 mM KCl, 1.1 mM $CaCl_2$, 0.6 mM $MgSO_4$, pH 7.3 with $NaHCO_3$). Unfertilized eggs were isolated from the ovarian lumen and placed into the saline within 2 h after ovulation.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated with ISOGEN (Nippon Gene) from various tissues, and single-stranded cDNA was synthesized from 1 μg of the total RNA by Superscript II (Stratagene) using Oligo$(dT)_{18}$ primer. Polymerase chain reaction (PCR) amplifications were carried out using primers [SPF, 5′-CACCATCAGCATGGAGCTG-3′ and SPR, 5′-AGGGTGTGTCCCATTTTCACATC-3′] to amplify a 348 bp fragment of alveolin cDNA. As a positive control of the experiment, the cDNA fragment was amplified from an alveolin cDNA clone plasmid, which was obtained from medaka ovarian cDNA library (Shibata et al., 2000), and as a negative control, same volume of water was added instead of the cDNA template. For standardization, a medaka actin cDNA fragment of 322 bp was amplified using specific primers [forward primer 5′-CACACCTTCTACAATGAGCTG-3′ and reverse primer 5′-CCGTCAGGATCTTCATGAGGT-3′]. Conditions for PCR were 1 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The PCR products were separated by electrophoresis on 1.5% agarose gels. For quantitative RT-PCR analysis of embryos and fry, sample preparation and measurement procedures were the same as described elsewhere (Shibata et al., 2010). For this experiment, the Qurt strain was used. In this strain, the genetic sex of the embryos can be identified by the presence (XY) and absence (XX) of leucophores on the head region from 2 days after fertilization (Wada et al., 1998). The primers for quantitative RT-PCR determination of alveolin transcripts were designed by Primer Express ver.2.0 (Applied Biosystems) and those are as follows: qAlv-F 5′-TCATCCCATACACCATCAGCA-3′; and qAlv-R 5′-TGATCTCCGACTGTCTGTGAGC-3′. Data were normalized by dividing the copy number values of alveolin by those of EF1α. All data are presented as means ± standard deviation (SD). Significant differences in the data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's posthoc test.

In situ hybridization

The cDNA fragment for alveolin was amplified using primers and inserted into pGEM-T Easy vector (Promega). The purified plasmid DNA was used as a template to generate a digoxigenin-labeled sense and antisense cRNA probe using a DIG RNA labeling Kit (Roche) according to the manufacturer's instruction. The ovaries were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4 °C and washed in PBS. Dehydration was done with methanol for 5 days at −80 °C. The ovaries were embedded in paraffin blocks and sectioned to 5 μm thickness. The procedures of in situ hybridization from hybridization to detection of signals were done according to the standard protocols as described elsewhere (Shibata et al., 2010).

Production of alveolin polyclonal antibody

The antigen, a synthetic peptide, CDIQKINTLYRCGSKY, corresponding to the C-terminal sequence of alveolin with an additional cysteine in the N-terminus was coupled to bovine serum albumin (BSA) with *N*-[ε-maleimidocaproyloxy]succinimide ester (EMCS, Pierce, Rockford, IL). The conjugate was injected into

BALB/c mice with Freund's complete or incomplete adjuvant (Wako, Japan) to generate polyclonal antibodies.

Immunohistochemistry

The freshly removed ovaries and eggs were rinsed with medaka saline, wiped to remove excess solution, placed into pre-chilled (−85 °C) methanol quickly and then kept for 5 days in an ultracold freezer (−85 °C). After this dehydration by methanol, samples were transferred to the refrigerator and further dehydrated using 100% ethanol twice. The samples were embedded in water-soluble resin, Technovit 7100 (Heraeaus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. After blocking with 10% goat normal serum, sections (1 μm in thickness) were incubated with an anti-alveolin antibody (1:50 diluted in PBS) overnight. The detection of signals was carried out with an alkaline-phosphatase conjugated anti-mouse IgG as secondary antibody (Zymed) and BCIP/NBT. Adjacent sections were counterstained with hematoxylin and stained by the periodic acid-Schiff (PAS) technique (Troyer, 1980). As a control, non-immunized mouse serum was used.

Preparation of cortical granules and egg exudates

The collection of isolated cortical granules was performed as described by Iwamatsu et al. (1995). One hundred unfertilized eggs were rinsed with Ca^{2+}-free saline (128.3 mM NaCl, 27 mM KCl, 6 mM $NaHCO_3$, 0.2 mM EDTA, 4 mM Hepes-NaOH, pH 7.0) and then centrifuged at 300*g* for 10 min. The eggs were placed in Ca^{2+} free saline in a glass depression slide and rapidly crushed and incised with a pair of fine forceps to disperse cytoplasmic inclusions. The glass slide was then agitated to gather the isolated cortical granules in the center. Pieces of the egg envelope (chorion) on the bottom and oil droplets on the surface of the solution were removed using forceps and a small pipette.

The egg exudate was obtained from activated eggs in which egg envelope had been removed as previously described (Iwamatsu et al., 1995).

Analysis of protein

N-linked oligosaccharide chains were removed by treatment with peptide *N*-glycosidase F (PNGase F, Takara). Fifty oocytes of each size were collected and homogenized by sonication with 500 μl of 100 mM Tris–HCl (pH 8.5) containing 10 mM EDTA. These samples were mixed with an equal volume of the denaturation buffer (1 M Tris–HCl, pH 8.5, 1% SDS and 0.2 M 2-mercaptoethanol), followed by boiling for 5 min. A 10 μl aliquot of each sample was added to 10 μl of 5% Nonidet P-40 and 2 μl of 0.5 unit/ml PNGase F, and then incubated for 20 h at 37 °C.

For immunoblotting, samples were subjected to SDS-PAGE, and separated proteins were electroblotted onto a PVDF membrane (Immobilon-P, Millipore). The blot was blocked with 5% nonfat dried milk in TTBS (50 mM Tris–HCl, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The blot was then incubated for 2 h in TTBS containing a specific antibody against alveolin (1:1000 diluted) and rinsed three times for 5 min in TTBS. The blot was then incubated for 1 h with a horseradish peroxidase-conjugated goat anti mouse IgG antibody (Zymed) in TTBS and rinsed three times for 5 min in TTBS. The rinsed blot was immersed in a chemiluminescence reagent (Perkin Elmer Life Sciences) and exposed to X-ray film.

To determine the N-terminal sequence of pro-alveolin, proteins extracted from previtellogenic oocytes were separated by 2D-PAGE. One hundred previtellogenic oocytes (400 μm diameter) were collected and deglycosylated protein samples were prepared as described above. After TCA precipitation and acetone wash, the sample was dissolved in homogenization buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT and protease inhibitors). Following alkylation by iodoacetoaminde, the sample was divided into two and subjected to 2D-PAGE according to manufacturer's instruction (Ettan IPGphor II and Multiphor II system, Amersham Bioscience). One gel was used for western blotting to identify the alveolin spots and other one was stained with SyproRuby to facilitate collection of the identified spots. N-terminal amino acid sequences were determined with an automatic sequencer (model 477 A, Applied Biosystems).

Phylogenetic analysis

A phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura et al., 2011). The amino acid sequences from first conserved tryptophan to last conserved cysteine of the protease domain of astacin family members were used. The alignment was performed using MUSCLE. Phylogenetic trees were constructed using maximum likelihood (ML) methods or neighbor-join (NJ) methods with 1000 Bootstrap Replications. The GenBank accession number of the aligned amino acid sequences are as follows: mouse astacin-like metalloendopeptidase (ovastacin), NP_766127; medaka six-cysteine containing astacin proteases (mc6ast2, NP_001098207.1; mc6ast3, NP_001098331; mc6ast4, NP_001098208; mc6ast5, NP_001098332; nephrosin, NP_001098206); medaka hatching enzymes (HCE1, NP_001188427; HCE2, NP_001098293.1; MAHCE, NP_001098205; LCE, NP_001098292); zebrafish six-cysteine containing astacin proteases (zc6ast1, NP_001036784; zc6ast3, NP_001013544; zc6ast4, NP_001020351; nephrosin, NP_991319; HCE-like, XP_001342763); zebrafish hatching enzymes (HE1a, NP_001038639; HE1b, NP_998800; HE2, NP_001091658); *Xenopus laevis* hatching enzyme (XHE, NP_001081221), Japanese quail hatching enzyme (QHE, BAD95472); mouse BMP1, NP_033885; zebrafish BMP1a, NP_001035126; zebrafish BMP1b, NP_001034901; mouse TLL1, NP_033416; zebrafish TLL1, NP_571085; mouse TLL2, NP_036034; mouse MEP1a, NP_032611; zebrafish Mep1a.1, NP_001025452; zebrafish Mep1a.2, NP_001122199; mouse MEP1b, NP_032612; zebrafish Mep1b, NP_001070089.

Several teleost genes which showed high similarity to alveolin (NP_001098139) were identified from expressed sequence tag (EST) databases. Their GenBank accession numbers are as follows: Senegalese sole (*Solea senegalensis*), FF286100; fugu (*Takifugu rubripes*), CA847215; stickleback (*Gasterosteus aculeatus*), CD507835; Coho salmon (*Oncorhynchus kisutch*), ES555394. Nas-15-like of zebrafish (XP_001337538) and a medaka EST sequence (DK226772) also showed high sequence similarities to alveolin. Their translated amino acid sequences predicted by the software, ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), were used.

Results

Tissue-specific expression of alveolin mRNA

The presence of alveolin mRNA in various tissues of adult medaka was examined by RT-PCR. A single band was only detected in the ovarian preparation (Fig. 1A) which showed the same size with those of the band in positive control. There were no signals from testis, brain, heart, liver, kidney, muscle, spleen or intestine, suggesting that the alveolin transcript is specifically expressed in the adult ovary.

Expression of alveolin mRNA during oogenesis

To identify the stages when alveolin is expressed and the cellular localization of alveolin mRNA in the adult ovary, in situ hybridization

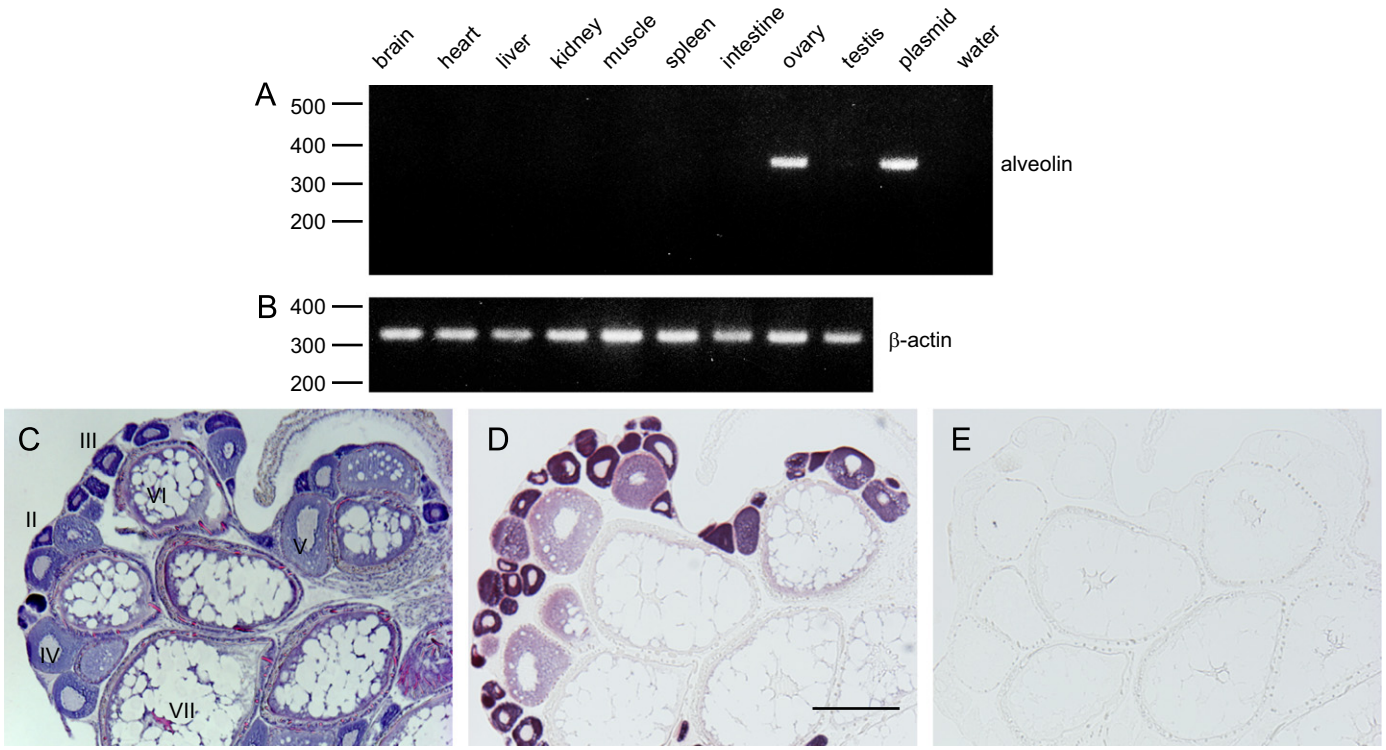


Fig. 1. Expression of alveolin mRNA in adult medaka. (A) Tissue distribution of alveolin analyzed by RT-PCR. The 348 bp PCR product was amplified from various tissues of adult medaka using alveolin specific primers. (B) As a control, a 322 bp cDNA fragment of cytoplasmic actin was amplified in each tissue sample. Numbers on the left side indicate the size of standards (bp). (C)–(E) In situ hybridization analysis in the ovary. Sections of medaka ovaries were hybridized with antisense probe (D) or sense probe (E), or stained by hematoxylin–eosin (C). Numbers indicate developmental stages of medaka oocytes as defined by Iwamatsu et al. (1988) (Supplemental Table S1). Bar in D indicates 500 μm.

was performed (Fig. 1C–E). Medaka, which spawns every day, has an ovary which contains oocytes undergoing all stages of oogenesis. The developmental stages of the oocytes were determined by the size and basic morphological characteristics according to Iwamatsu et al. (1988) (Supplemental Table S1). In the previtellogenic phase (stages I–IV), cortical granules had not yet formed but the strongest signal was observed in the cytoplasm of the smaller oocytes (stage II; early previtellogenic phase). In the vitellogenic phase (stages V–VIII), the signal became weak. These results show that alveolin is expressed in the oocyte and expression begins at a very early stage of oogenesis.

The expression level of alveolin mRNA at each oocyte stage was quantified relatively by quantitative RT-PCR (Table 1). Aspects of the results of quantification appear to be different from those of in situ hybridization. The relative expression levels of alveolin mRNA is not significantly different throughout the previtellogenic and vitellogenic stages examined. This difference is probably due to the in situ signal appearing weak resulting from the diffusion of the transcripts into the enlarged cytoplasm. The amount of alveolin mRNA decreased in mature oocyte (unfertilized egg: stages X). These quantitative RT-PCR results indicate that the alveolin mRNA expression is maintained at a similar level throughout the vitellogenic phase until the maturation stage.

To further identify when alveolin expression starts in oogenesis, the expression was examined in embryo and fry stages. The expression levels were measured by quantitative RT-PCR at 2-day intervals from 2 to 14 days post-fertilization (dpf) (Fig. 2A). A significant increase in expression of alveolin mRNA was observed at 14 dpf (6 days after hatching), in a female specific manner. In situ hybridization confirmed that transcripts were already localized in the oocytes at 14 dpf (6 days after hatching) (Fig. 2B). At 30 days after hatching, the developed ovary contains many oocytes which were positive to the alveolin probe (Fig. 2C). The results suggest that alveolin transcription may start immediately after oogonia differentiate into oocytes.

Table 1
Relative expression levels of alveolin mRNA in each stage oocyte.

Diameter of oocyte (μm)	Stage	Expression level ($\times 10^2$)
75	II	12.11 ± 2.88^a
150	IV	12.07 ± 4.01^a
250	V	11.98 ± 1.62^a
400	VI	12.09 ± 3.23^a
500	VII	11.43 ± 2.26^a
750	VIII	11.26 ± 2.73^a
900	IX	7.07 ± 0.44^a
1200	X (unfertilized egg)	1.26 ± 1.39^b

The relative expression level represents the ratio of copy number of alveolin to that of ef1α. Each value is represented as mean ± standard deviation (SD), $n=3$. Values sharing the same superscript do not vary significantly from one another. The expression level at stage X was significantly lower than all other values ($P<0.01$).

Immunohistochemical observations of alveolin protein

To identify where alveolin protein is localized, sections of ovaries and eggs were immunostained with the alveolin antibody (Fig. 3C and F). Adjacent sections were also stained by hematoxylin–eosin, the PAS reaction and non-immune serum (Fig. 3A, B and D). Fish cortical granules are strongly stained by the PAS reaction due to the high polysaccharide content. Yolk spheres were also positive for the PAS reaction, but could be distinguished from cortical granules by their heavy staining with hematoxylin (Fig. 3A and B). Only the cortical granules were intensively stained by antibody in the oocytes (Fig. 3C) and unfertilized eggs (Fig. 3F). Notably, dense cores contained in cortical

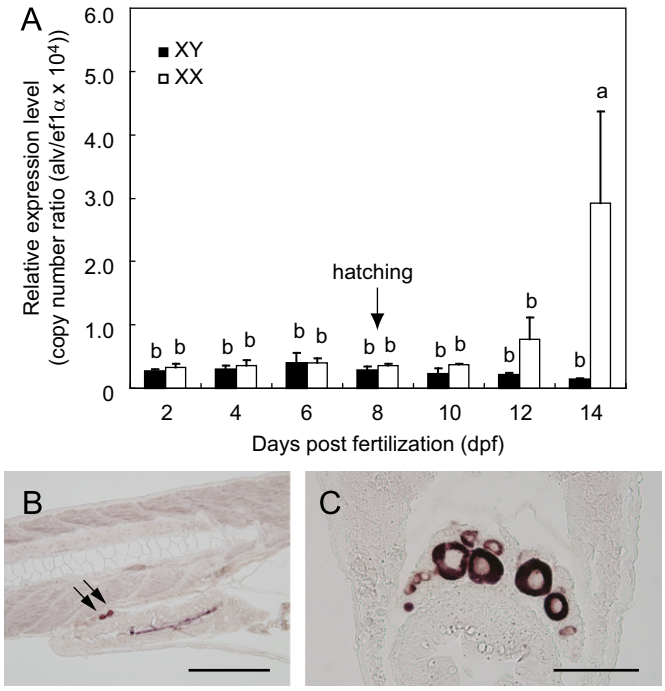


Fig. 2. Expression of alveolin mRNA in medaka embryos and fry. (A) The expression levels were measured by quantitative RT-PCR at 2-day intervals from 2 to 14 days post-fertilization (dpf). Almost all embryos hatched at 8 dpf. As shown in Fig. 2A, a significant increase in alveolin expression was detected in XX embryos at 14 dpf (6 days after hatching) ($P<0.01$, $n=3$). The bars sharing the same superscript are not significantly different from each other. Values from males did not vary significantly from one another. (B) In situ hybridization showing expression of alveolin in the gonads of 14 dpf (6 days after hatching) XX fry in sagittal sections. Arrows indicate the oocytes (stage I) displaying hybridization signal in the gonad. Bar shown in (B) is 200 μm. (C) In situ hybridization showing expression of alveolin in the gonads of 30 days after hatching (hatching was occurred at 8 dpf) XX fry in transverse sections. Bar shown in C is 100 μm.

granules seemed to be strongly stained by both the antibody and the PAS reaction. The first appearance of cortical granules occurred during the early vitellogenic stage (stage V; 151–250 μm in diameter). Prior to vitellogenesis, they increased in number and size and filled the cytoplasm at stage VII (The yolk is not yet seen in the oocytes which was labeled as VII in Fig. 3A–D). Cortical granules were displaced to the periphery of the oocyte as a result of the accumulation of massive amounts of yolk in the central region of the oocyte during stage VIII. After oocyte maturation, cortical granules were located in the cortex region as a single layer. The results indicate that alveolin is synthesized and stored in cortical granules, concomitant with cortical granule biogenesis.

Synthesis of alveolin during oogenesis

The stage-dependent appearance and changes in the relative level of pro-alveolin during oogenesis was analyzed by western blotting (Fig. 4). As mentioned later, pro-alveolin in unfertilized eggs was detected as a broad band, due to its *N*-glycosylation. To distinguish any small differences between protein bands visualized by SDS-PAGE, the samples were deglycosylated by treatment with PNGase F before electrophoresis. Samples equivalent to one oocyte from each stage were applied to individual lanes on the gel. In stage IV oocytes, no signal was detected. The 32 kDa band was first detected in stage V oocytes and increased in intensity in stage VI and VII oocytes. Following the appearance of the 32 kDa band, the 30 kDa band was detectable in stage VI oocytes as a very faint signal, and which increased in intensity during stage VII and VIII. The 32 kDa band continued to be present, but the relative amounts of 32 and 30 kDa bands reversed in stage VIII oocytes. In the mature, unfertilized egg, only the 30 kDa band was detected. These two bands of 32 and 30 kDa were also observed in the samples prepared from isolated cortical granules of vitellogenic stage oocytes (Supplemental Figure S1).

To clarify the difference between the 32 and 30 kDa forms of alveolin, their N-terminal sequences were analyzed. The protein extracted from previtellogenic oocytes of 400 μm diameter was separated by 2D-PAGE after removal of N-linked glycosides and the spots of the 32 and 30 kDa forms were identified by western blotting (Supplemental Figure S2). Unexpectedly, each band was separated by SDS-PAGE into further two spots with different isoelectric points. These four proteins were eluted from excised gel spots and the N-terminal amino acid sequences were analyzed. N-terminal sequence of the two 32 kDa spots and the two 30 kDa spots were identical, respectively. The N-terminal sequence of both 32 kDa spots was determined as APVPSTQEAFKSVPGV, just corresponding to the signal peptide cleavage site predicted by the computer program (SignalP; http://www.cbs.dtu.dk/services/SignalP/). The N-terminal sequence of the two 30 kDa spots was determined as AFKSVPGVDSTATDQ, indicating that the eight amino acids (APVPSTQE) of N-terminus of the 30 kDa form has been posttranslationally truncated from those of the 32 kDa form. These results suggest the possibility that synthesized alveolin protein is accumulated in cortical granules after removal of signal sequence and then underwent further truncation in the N-terminus within the cortical granules during oogenesis. In the present study, although the 32 and 30 kDa proteins were separated by 2D-PAGE into further two spots (Supplemental Figure S2), any difference between each two spots (same molecular mass but different isoelectronic points) was not detected. It is conceivable that these differences are made by some posttranslational modification on same protein backbone.

The Asn^{35}, Asn^{40} and Asn^{52} residues in alveolin precursor are predicted as *N*-glycosylation sites by the sequence context of Asn-Xaa-Ser/Thr sequons (Shibata et al., 2000). The amino acid sequencing of the N-terminus of the 30 kDa form confirmed the first two *N*-glycosylation sites. These two Asn residues in the 30 kDa form corresponding to Asn^{35} and Asn^{40} of alveolin precursor were detected as Asp residues because their glycosylated side chains were converted to a carboxyl group by hydrolytic cleavage during deglycosylation. In the present study, these Asn positions in 32 kDa form and the Asn^{52} in 30 kDa form could not be analyzed by N-terminal sequencing due to signal decay during the Edman degradation cycle.

Processing of alveolin associated with cortical granule breakdown

For characterization of pro-alveolin by western blotting, the cortical granules were isolated from unfertilized eggs with Ca^{2+}-free saline to suppress autolysis. In the cortical granule suspension sample, a positive band was detected with an apparent molecular mass of 50 kDa on SDS-PAGE. When 40 μl of the hypotonic solution (50 mM Hepes-NaOH, 1 mM $CaCl_2$, pH7.5) was added to a 10-μl aliquot of the cortical granule suspension, most granules burst within 1 min with relatively few granules remaining intact (Fig. 5A and Supplemental Movie S1). In the burst cortical granules sample, the immunoreactive band showed an apparent molecular mass of 21.5 kDa, which is same size as those of alveolin in egg exudates. By contrast, the positive band in intact cortical granules was changed from a 50 kDa broad band to a 30 kDa clear band after PNGase F treatment, indicating the presence of the N-linked sugar chains. These results indicate that cortical granules of mature eggs contain a pro-alveolin modified with the N-linked sugar chains. It is likely that the processing of pro-alveolin which occurs at the time of fertilization was mimicked in vitro during artificial bursting of the isolated cortical granules.

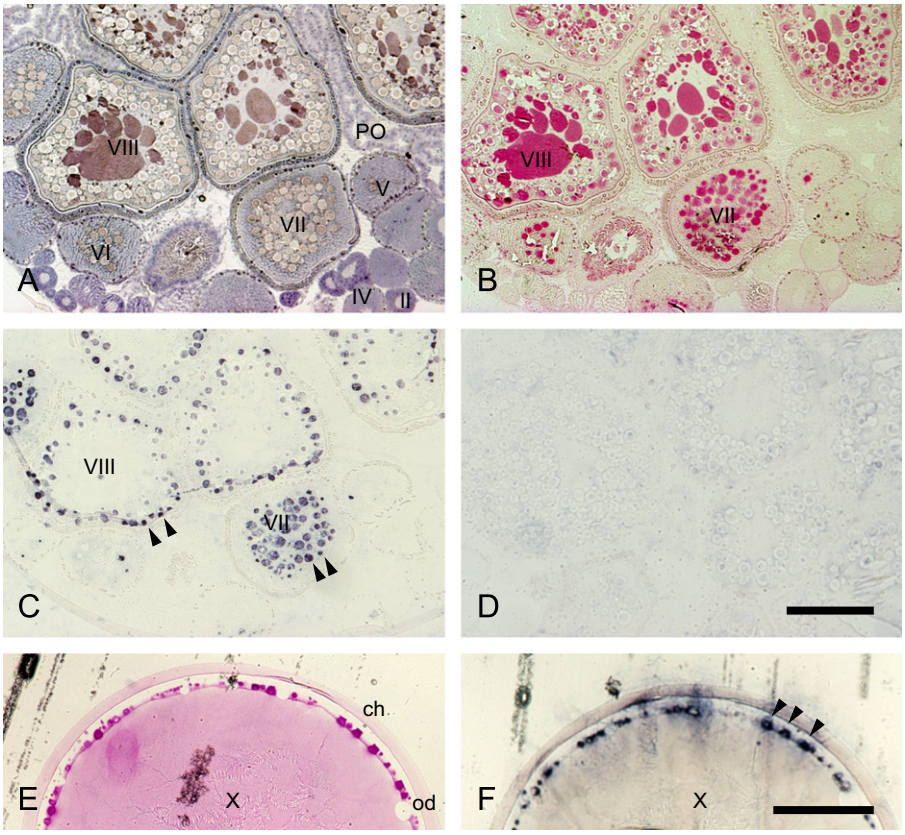


Fig. 3. Immunolocalization of alveolin within ovary and unfertilized eggs. Sections were stained with anti-alveolin antibody (C) and (F) or non-immune serum (D). For histological observation, sections were stained with hematoxylin (A) and by the periodic acid-Schiff reaction (B) and (E). The typical alveolin signals are indicated by arrowheads in (C) and (F). Numbers in the figures indicate developmental stages of oocytes (the stages are summarized in Supplemental Table S1). PO, postovulatory follicles; ch, egg envelope (chorion); od, oil droplet. Bars shown in (D) and (F) are 200 μm.

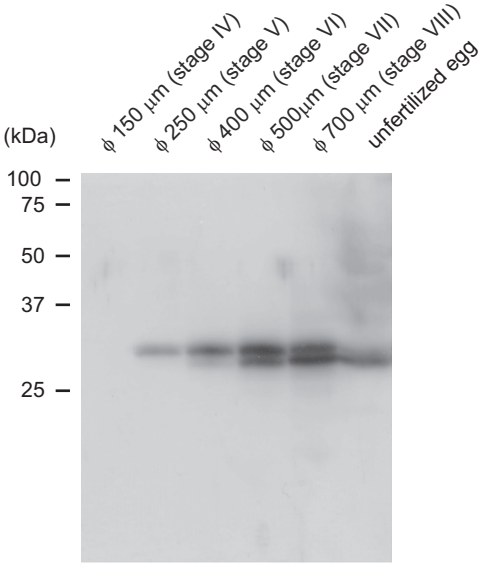


Fig. 4. Synthesis and accumulation of alveolin protein during oogenesis. Extracts of oocytes at various stages were deglycosylated by PNGase F and subjected to immunoblotting. Upper band is 32 kDa and lower band is 30 kDa. Numbers at the top indicate the size of oocytes in diameter and oocyte developmental stages. Numbers on the left side indicate molecular masses of standards (kDa).

The cortical granules isolated from immature oocytes (stage XIII, 600 μm in diameter), which are not yet ready for exocytosis, were also examined (Supplemental Figure S1). In immature oocytes, cortical granules contain both 32 kDa and 30 kDa form of pro-alveolin. When the hypotonic solution was added to the isolated cortical granule suspension which was being maintained in Ca-free saline, the isolated cortical granules started bursting and alveolin was processed into 21.5 kDa form, the same size as that observed with mature oocytes. This result suggests that the machinery for processing of pro-alveolin have already been within cortical granules of immature oocytes.

Inhibition of processing of a 50 kDa band in the presence of inhibitors

Transformation of the 50 kDa band to a 21.5 kDa band was observed after breakdown of isolated granules (Fig. 5). The in vitro breakdown experiment was performed in the presence of protease inhibitors (Fig. 6A). In the presence of 4 mM AEBSF or 10 μg/ml leupeptin, the cortical granules burst the same as controls, but the 50 kDa bands did not change. A similar result was obtained with high concentrations of EDTA.

To assess the relationship between the pro-form and activity, the isolated egg envelope was incubated with the supernatant of the burst cortical granules by the same method reported previously (Iwamatsu et al., 1995). To obtain the mature form of alveolin, cortical granules were burst without leupeptin and then leupetin stock solution was added to make a final concentration of 10 μg/ml. This sample showed the 21.5 kDa form in western blotting and this form cleaved ZPBs and polymerized ZP proteins in the incubated egg envelope (Fig. 6B). The pro-form of alveolin was obtained by bursting cortical granules in the presence of 10 μg/ml leupeptin. The pro-alveolin sample did not induce changes in the egg envelope protein. The results suggest that pro-alveolin is a latent form without biological activity.

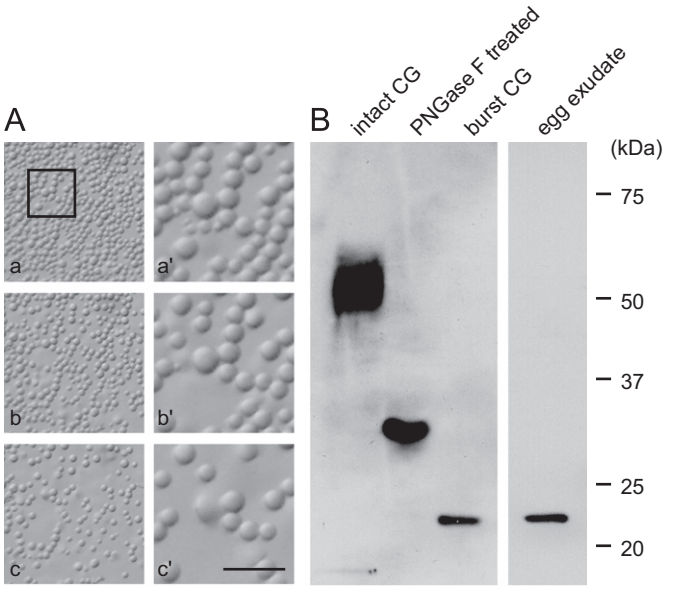
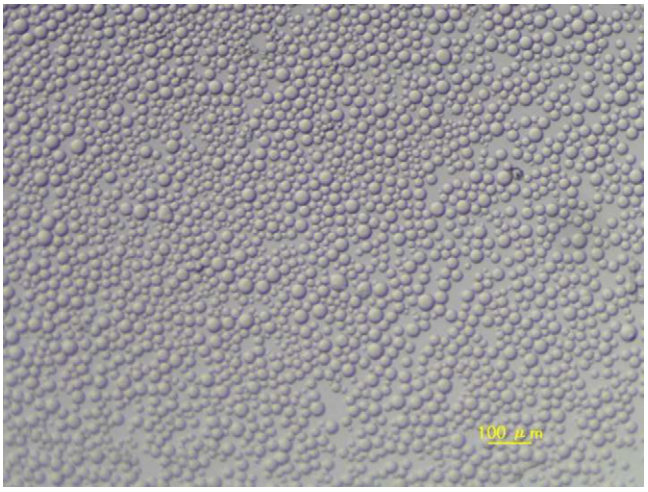


Fig. 5. In vitro breakdown of isolated cortical granules and processing of alveolin. (A) Parts (a)–(c) show sequential photographs of isolated cortical granules during bursting (see Supplementary Movie S1). Cortical granules were isolated from unfertilized eggs and maintained in Ca-free saline. Granule breakdown started immediately after addition of hypotonic solution and finished within a few minutes. (a) 10 s; (b) 15 s; (c) 20 s after adding hypotonic solution. Parts (a′)—(c′) are high magnification photographs in areas marked by squares in (a). Bar shown in (c′) is 100 μm. (B) Changes in molecular mass of alveolin were analyzed by immunoblotting before and after granule breakdown. Isolated cortical granules showed a broad band of 50 kDa. PNGase F treatment (as described in the Materials and methods section) reduced the size of the 50 kDa band to 30 kDa. After breakdown of the cortical granules, a bands of 21.5 kDa was revealed, the same size as in exudates. Numbers on the right side indicate molecular masses of standards (kDa).



Movie S1. A video clip is available online. Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2012.09.016.

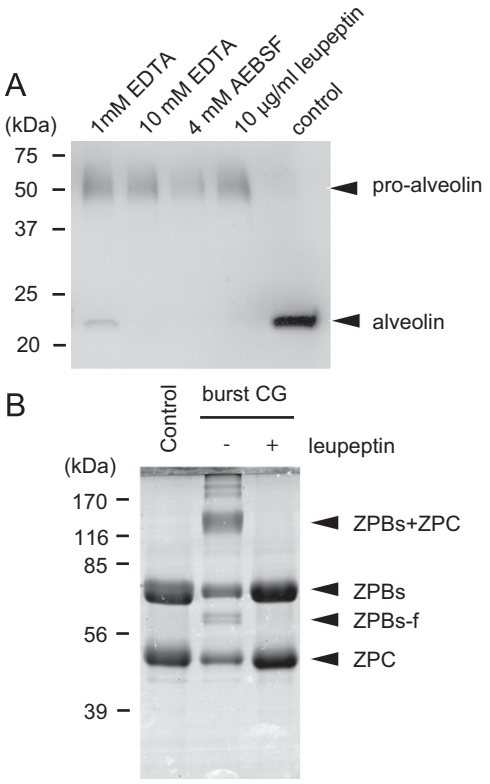


Fig. 6. Effects of leupeptin, AEBSF and EDTA on transformation of alveolin. (A) Western blot of alveolin shows that the conversion of the 50 kDa band to 21.5 kDa was suppressed in the presence of inhibitors. Inhibitors were dissolved in 50 mM Hepes-NaOH, 1 mM CaCl2 (pH 7.5) and added at concentrations of 10 μg/ml for leupeptin, 4 mM for AEBSF and 1 and 10 mM for EDTA. (B) The activities of contents of cortical granules which were burst in the presence or absence of leupeptin were tested. The isolated egg envelopes were incubated with burst cortical granules at 27 °C for 30 min. The cortical granules which were burst in the absence of leupeptin, and then mixed with a small volume of leupeptin caused cleavage of ZPB (converted into ZPBf) and polymerization of ZP proteins (ZPBs+ZPC). The cortical granules which were burst in the presence of leupeptin did not cause any changes in ZP proteins. Numbers on the left side indicate molecular masses of standards (kDa).

Phylogenetic analysis of vertebrate astacins

To compare alveolin with other major astacin family members of vertebrate, phylogenetic analysis was performed. As the representative Meprins and BMP1/Tolloids of vertebrates, zebrafish and mouse protein sequences were used. It was found that medaka also has orthologs for each Meprin and BMP1/Tolloid member based on genome database search, but the medaka cDNA sequences have not yet been determined. It is well known that amphibians and birds have the astacin-like hatching enzyme. *X. laevis* and Japanese quail hatching enzyme sequences were added to make the phylogenetic tree. The phylogenetic trees were constructed by the maximum likelihood method and the neighbor-join method by MEGA 5 software and were essentially the same. Only the result from the maximum likelihood method is shown here (Fig. 7). Teleostean fish have astacin-like hatching enzymes and their paralogs. All of them are characterized by two extra cysteine residues in addition to the four cysteines conserved in astacin family members and are tentatively named as six-cysteine containing astacin proteases (C6AST) (Kawaguchi et al., 2006). In this phylogenetic analysis, a node of the hatching enzyme group, including C6ASTs, *X. laevis* hatching enzyme, Japanese quail hatching enzyme and mouse ovastacin, showed a low bootstrap value due to their sequence divergence. Meprins and BMP1/Tolloids groups formed distinct clades. Although alveolin, its fish orthologs and nas-15-like showed long branches, they formed a group with a node having a high bootstrap value, suggesting that alveolin does not belong to any known groups in the vertebrate astacin family. In the present study, we could not find apparent alveolin orthologs in tetrapods.

Discussion

At the time of fertilization, the contents of egg cortical granules are released into the perivitelline space, between the egg and the egg

envelope. A long-held idea is that the hardening of the fish egg envelope is caused by some substance released from cortical granules (Nakano, 1956). Zotin (1958) first identified the existence of some thermally-labile substance in perivitelline fluid of freshly activated salmonid eggs, which was able to harden the egg envelope. He speculated that 'hardening enzyme' is released from the egg surface and initiates the polymerization reaction of egg envelope components. In the previous study, we had identified the egg envelope hardening initiation factor, alveolin, which was discharged from the egg at the time of egg activation (Shibata et al., 2000). Here, using a specific antibody against alveolin, we showed that alveolin is synthesized during oogenesis, packaged into cortical granules as a glycosylated latent pro-form and released upon fertilization along with its activation by removal of the pro-domain. Fig. 8 represents a hypothesized pathway for synthesis, accumulation and secretion of alveolin revealed by studies of the medaka model.

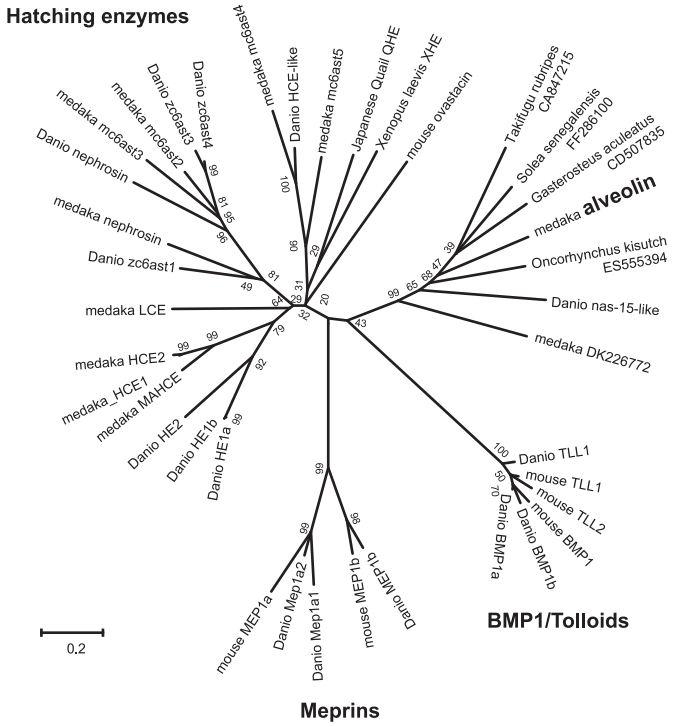


Fig. 7. A phylogenetic tree of major members of vertebrate astacin family. The phylogenetic tree illustrates the evolutionary relationships among Hatching enzymes, BMP1/Tolloids, Meprins groups and alveolin, including homologs in some teleosts.

In the previous study, the purification of alveolin was accomplished through the combination of three kinds of chromatography (anion exchange, hydrophobic interaction and gel filtration chromatography) of egg exudate with two types of assay: the egg envelope protein polymerization, and the cleavage of ZPB protein. At each step from the exudate to the final single protein fraction, both activities were always detected in same fractions. Therefore, we concluded that the processing of ZPB protein by alveolin causes the reassembly of egg envelope proteins and triggers the TGase reaction to form cross-linking among the ZP proteins. Alveolin is a member of the astacin metalloprotease family and was proposed to be a protease which hydrolyzes ZPB at a specific site. Teleosts (Yasumasu et al., 1992, Inohaya et al., 1997), amphibians (Katagiri et al., 1997) and birds (Yasumasu et al., 2005) utilize astacin-like proteases as their hatching enzymes. Although both of these astacin family proteases target the egg envelope proteins in medaka, they have opposing functions. Whereas hatching enzymes (HCE1, HCE2 and LCE) digest the egg envelope protein for hatching, alveolin induces reassembly of ZP proteins to make the egg envelope rigid at the time of fertilization. The phylogenic analysis of astacin family members demonstrates the uniqueness of alveolins among the vertebrate astacin family members: they are phylogenetically separated from the hatching enzyme group and this likely reflects differences in structure and substrate specificity.

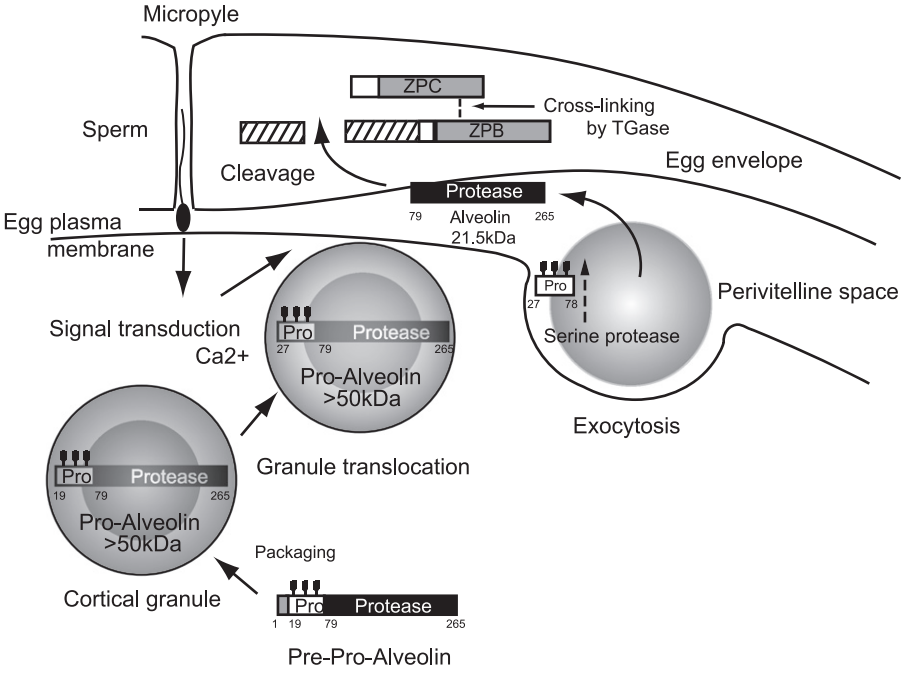


Fig. 8. A hypothesized pathway for alveolin from synthesis and accumulation to secretion. Alveolin is synthesized as a pre-pro form and accumulates in cortical granules as a pro-form throughout oogenesis. During the oocyte maturation stage, cortical granules containing pro-alveolin translocate to beneath the plasma membrane. Upon fertilization, pro-alveolin is processed to an active form and released into the perivitelline space. Alveolin hydrolyzes the N-terminal region of ZPB. Then, transglutaminase (TGase) starts to catalyze the polymerization reaction between ZPB and ZPC with intermolecular cross-linking. Hatched region in N-terminus of ZPB represents repetitive domain. Gray regions of ZPB and ZPC are ZP domains.

Here, we revealed that alveolin is a cortical granule-derived astacin family protease which is specifically expressed in oocytes. In human and mouse, the oocyte-specific expression of an astacin-like proteases, tentatively named ovastacin, has also been described (Quesada et al., 2004). Although it had been thought that ovastacin is a mammalian homolog of fish hatching enzymes based on phylogenetic analysis (Quesada et al., 2004, Kawaguchi et al., 2007), a recent report demonstrated that ovastacin is stored in egg cortical granule and is released to cleave ZP2 protein at the time of fertilization and this is results in the prevention of polyspermy (Burkart et al., 2012). On the other hand, the medaka genome contains several paralogous genes of hatching enzyme, which are thought to be generated through gene duplication from single ancestral hatching enzyme (Kawaguchi et al., 2007). However, none of these genes exhibit oocyte-specific expression (Kawaguchi et al., 2006). It is conceivable that alveolin is not a direct ortholog of ovastacin, but alveolin and ovastacin shows similarities in their expression patterns and biological function. The female-specific expression of alveolin in medaka was first reported by Kanamori (2000). The cDNA fragment encoding the alveolin transcript (choriolysin L-related, GenBank ID: AF72191) was identified in female fry 30 days after hatching by subtractive hybridization screening. We could detect alveolin mRNA expression in females at 14 dpf (6 days after hatching), confined to the oocyte. In medaka, the transition from primordial germ cells to oogonia is observed in the gonad of fry on the first day after hatching, and the transition from oogonium to oocyte is observed in the ovary of fry which had developed at 22 ± 2 °C at 5 days after hatching (Satoh, 1974). Considering the development dates of the fry, alveolin transcription may start just after oogonia differentiate into oocytes. Progression of gonadal development is variable in each medaka strain and with culture conditions. This suggestion needs to be verified by more precise investigation to determine the stage when alveolin transcription starts. Although alveolin acts at the time of fertilization, the mRNA expression starts at a very early stage of oogenesis and the expression level is then maintained at the same high level throughout oogenesis until the oocyte maturation stage. By contrast, the appearance of alveolin protein was delayed until the beginning of the vitellogenic phase (stage V; cortical granule formation stage). Thus, the translation of alveolin transcripts seems to be suppressed during the previtellogenic phase. Because ovarian follicle growth was arrested before stage V in hypophysectomized medaka (Iwamatsu and Akazawa, 1987), the starting point of alveolin translation likely implies the presence of some breakpoint during oocyte development controlled by pituitary hormones.

Alveolin possesses a domain organization composed of prepeptide (signal peptide), pro-domain and metalloprotease domain as commonly seen in all astacin family members, reflecting the features of extracellular proteases, and the purified active alveolin has only the metalloprotease domain as its mature form (Shibata et al., 2000). The present study revealed that alveolin packaged into cortical granule is in the pro-form until exocytosis upon fertilization. It is generally thought that astacin family enzymes are synthesized as inactive (latent) proenzymes, and removal of the prosequences constitutes a major mechanism for activation. Structural studies of proastacin show that the N-terminal pro-domain renders the astacin inactive by covering the active-site (Bode et al., 1992, Guevara et al., 2010). The present study shows that pro-alveolin lacks biological activity. Even though we showed the pro-form status of alveolin in unfertilized eggs by western blotting, the current in vitro examination is essentially similar to the previous in vivo examination carried out by Ha and Iuchi (1996) with regard to the use of leupeptin to suppress alveolin activation. In the present study, we showed that the processing of pro-alveolin was inhibited by the serine protease inhibitors/AEBSF and leupeptin. Because the processing occurs on the C-terminal side of basic amino acid residues in most members of the astacin family, tryptic activity has been thought as one factor participating in this processing. Alveolin also has an arginine residue in that position, supporting the possibility of involvement of tryptic activity. The requirement of serine proteases for activation of ZPA protease has been reported in *X. laevis* (Lindsay and Hedrick, 1989). Among them, chymotrypsin-like protease had been localized in the perivitelline space of unfertilized egg (Lindsay et al., 1992). In contrast, because the cortical granules isolated from immature oocytes also exhibit the processing of pro-alveolin during bursting (Supplemental Figure S1), the dormant processing protease seems to co-exist with alveolin in cortical granules throughout oogenesis until exocytosis. Similarly, polysialoglycoprotein (PSGP) and the protease, PSGPase, which cleaves the PSGP in the perivitelline space following exocytosis upon fertilization, also co-exist in the cortical granules of rainbow trout (Kitajima and Inoue, 1988) and medaka (Kitajima et al., 1989) unfertilized eggs. Judging from the enzymatic properties of PSGPase, which does not have trypsin-like activity, it seems not to be the direct processing protease for pro-alveolin. Our preliminary gelatin zymography of the egg exudate showed three bands (data not shown). It is likely that several proteases are contained in the cortical granule. Further analysis is needed to identify the protease(s) involved in processing of pro-alveolin and to clarify the molecular mechanism involved in the processing. In the present study, we suggest the possibility that the N-terminal truncation of pro-alveolin (posttranslational processing from 32 to 30 kDa form) occurred during oogenesis within the cortical granules, but unfortunately we lack further information on biological significance of this cleavage at present.

The mechanisms of changes in chemical and physicochemical property of egg envelope upon fertilization have been well studied in *Xenopus* and mammals. The limited proteolysis of ZPA/ZP2 triggers conformational changes in egg envelope proteins and results in egg envelope hardening (Drobnis et al., 1988; Bakos et al., 1990). In *X. laevis*, the molecular identity of ZPA protease has not yet been identified. However, its enzymatic properties are identical to those of BMP-1, which is a member of the astacin metalloprotease family (Lindsay and Hedrick, 2004). There is a strong similarity between frogs and mammals in the mechanisms of egg envelope hardening. Although the information on the involvement of cortical granule proteases in other teleost fish is lacking at present, our studies on medaka fish showed that the basic mechanism of egg envelope hardening is similar to those of these higher vertebrates. However, the involvement of TGase in egg envelope hardening is a unique feature of fish. ZPA/ZP2 type of ZP gene has not yet been found in fish to date. Moreover, alveolin seems not to be a direct ortholog of ovastacin. These facts raise the possibility that the mechanism of egg envelope hardening of fish developed independently from those of tetrapods. In medaka, metalloprotease cleaves ZPB at the N-terminal side of the aspartic acid residue which is positioned in the Pro-Gln-X repetitive region upon fertilization (Ha and Iuchi, 1996). The presence of the repetitive domain in the N-terminal side of ZP protein is a unique feature of many teleost fish ZPBs and is sometimes seen in ZPCs. Although the pattern of the repeat varies in each species, generally it is rich in prolines and glutamines (Kanamori et al., 2003). Based on the abundance of glutamine, it has been postulated that this domain serves as a cross-linking site for the TGase reaction during egg envelope hardening. Involvement of TGase in egg envelope hardening had already been reported in some fish species but the involvement of alveolin in other teleosts is unknown. Because the existences of homologous genes of alveolin in some other teleosts were predicted from EST data base searching, it is expected that the

mechanism we describe is conserved in other teleost fish. The function of alveolin homologs in other teleost species needs to be determined.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.09.016.

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