Blocks to Polyspermy in Fish: A Brief Review

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Abstract

Although fertilization was observed by Fol more than 100 years ago, mechanisms for effecting a block to polyspermy in fish are largely unknown. In this review, the recent studies of polyspermy block mechanisms in teleost fish eggs will be presented, including morphological, biochemical, and molecular results.

Introduction

In the animal world, to survive and leave offspring, each species has evolved different mechanisms. In mammals, the upper reaches of the female's oviduct, the infundibulum, is the site of fertilization, and a special site to implant the embryo has evolved, the uterus, where subsequent development /nutritional support takes place. After implantation in the uterus the mammalian fetus develops the respiratory, excretory and nutritive supply line directly to the mother, the placenta. Another special organ is the mammary gland to feed the newborn on mammary secretions. Compared with other species, mammals are able to give birth to a small number of offspring at one time because their guardian system for embryo growth and parental care including lactation provides increased safety and augments the likelihood of continued development for the most vulnerable stages (Jameson, 1988).

In contrast to mammals, oviparous fish have developed a system to spawn a huge number of eggs and to fertilize them at the same time. Before spawning, during oogenesis in the female, the vitellogenin and choriogenin are synthesized in the liver under the influence of estrogen and accumulate in the oocyte as the yolk to serve as nutrition for the embryo, and in the egg envelope (chorion) to protect the embryo from the chemical, physical and biological pressure in the environment (Yamagami et al., 1994). While the parents produce a huge number of fertilized eggs at one time, the number of embryo to survive to maturity is very small. Under natural conditions, fertilization must be completed between one maternal gamete (oocyte, egg) *vs.* one paternal gamete (sperm). It is fatal if more than one sperm fuses with the oocyte, and most embryos die at early stage. During fertilization, a huge number of sperm compete with each other to fertilize an oocyte. Among the fish except for the sharks and chimaeras, only a single sperm penetrates into the cytoplasm of the oocyte. In the case of sharks and chimaeras, more than one sperm normally enters the oocyte, while only one sperm nucleus fuses with the oocyte nucleus, although the mechanisms controlling these physiological polyspermic fish eggs is unclear. For monospermic eggs, mechanisms to prevent supernumerary sperm penetration into the oocytes, called “the block to polyspermy or polyspermy block,” must exist, and to date a large part of these mechanisms remains unclear. In this review, the current understanding of major strategies employed by the fish egg to block polyspermy will be discussed.

Comparison between the mammalian fertilization and oviparous fish fertilization

Historically, many investigators reported the importance of an intact egg envelope (chorion, vitelline envelope, zona pellucida, zona radiata) to maintain monospermic fertilization in the fish egg. When the egg envelope is removed from the unfertilized egg followed by insemination, the egg always becomes polyspermic (Kanoh and Yamamoto, 1957; Yanagimachi, 1957; Sakai, 1961; Iwamatsu, 1983; Iwamatsu and Ohta, 1978; Ginzburg, 1961; Gamo et al., 1960). In oviparous fish eggs, there is the unique structure called the micropyle on the surface of the egg envelope where the sperm can only enter to go through and attach to the oocyte plasma membrane; this structure does not exist in the mammalian egg.

Figure 1 shows a comparison of the fertilization process between mammals and oviparous fish. In mammals, to succeed in fertilization, sperm must first bind to the egg envelope, the zona pellucida (**Fig.1a**). The egg envelope of mammals consists of at least three major glycoproteins, ZPA, ZPB and ZPC. Since 1985 when Florman and Wassarman identified ZPC as the sperm receptor, specifically its O-linked carbohydrate chains, many investigators have studied the molecular mechanisms of sperm-egg interaction in mammals.

After the sperm bind to ZPC, a component of the sperm acrosome, located at the sperm head, called "acrosin," is released to digest the egg envelope (zona pellucida) glycoproteins to make it possible for fusion between the sperm plasma membrane and oocyte plasma membrane (acrosomal reaction, **Fig.1b**). In 1988, Bleil et al identified ZPA is the secondary sperm receptor for sperm during the fertilization process in mice to maintain the binding of acrosome-reacted sperm to the egg ZP during penetration. The attachment of the sperm plasma membrane to the oocyte plasma membrane induces the Ca^{2+} wave in the egg to the opposite site from the sperm-egg attachment site, following the release the contents of the egg cortical granules (cortical reaction). During the cortical reaction, the contents of the cortical granules interact with the egg envelope (zona reaction) and no more sperm can penetrate into the oocyte.

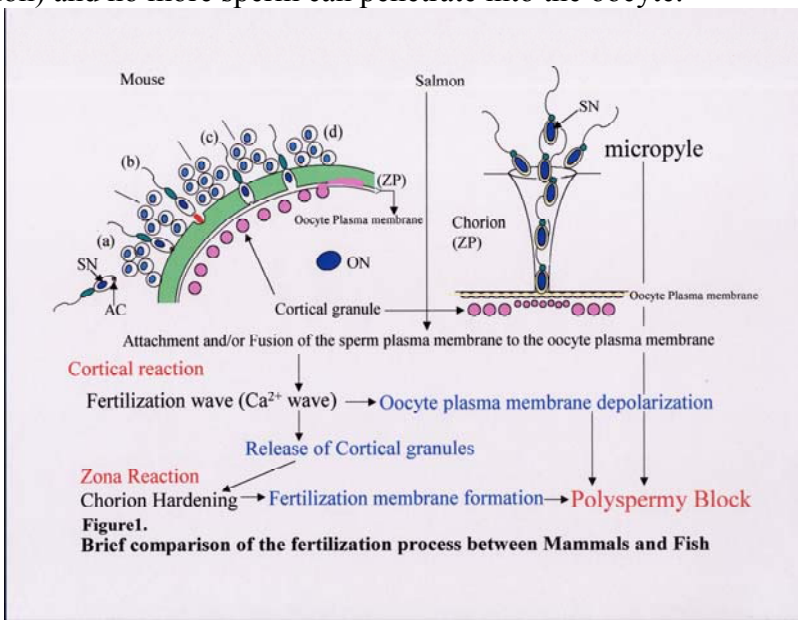

Figure 1. Brief comparison of fertilization process between mammals and fish
SN; sperm nucleus, AC; acrosome, ON; oocyte nucleus, ZP; egg envelope (zona pellucida, chorion)

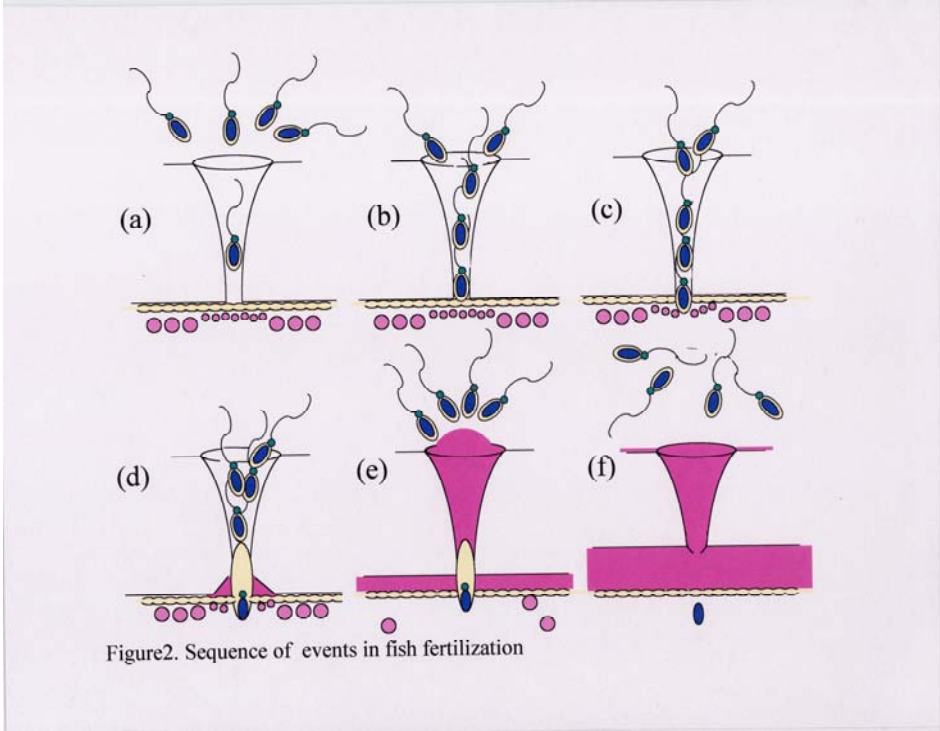

Figure2. Sequence of events in fish fertilization

Figure 2 shows the summary of the mechanisms of polyspermy block in fish egg:

(1) The diameter of the inner aperture of the micropyle restricts the number of sperm entering the micropyle. (**Fig.2a**)

(2) The fertilization cone prevents excess spermatozoa from attaching to the oocyte plasma membrane and/or from entering into the perivitelline space. (**Fig.2b, 2c**)

(3) Cortical granule lectins immobilize the excess sperm in the micropylar vestibule. (**Fig.2d, 2e**)

(4) The perivitelline fluid containing cortical granule lectins as the sperm agglutinin flows out along with excess spermatozoa through the micropylar aperture, caused by the increase of osmotic pressure. (**Fig.2e**)

(5) Perivitelline fluid containing cortical granule lectin through the micropyle interacts with the outside of the fibril layer to eliminate sperm guidance and attractant to the micropyle. (**Fig.2f**)

(6) Hardening of the chorion by alveolin and transglutaminase following inner layer chorion shrinkage decreases the diameter of the micropyle or entirely closes the micropyle resulting in shutting out of supernumerary sperm. (**Fig.2f**)

Compared with the process of mammalian fertilization, fish oocytes have the micropyle that consists of a vestibule and a micropylar canal, which is generally funnel-shaped (see detail below). The sperm can attach to the oocyte plasma membrane only through the micropyle, therefore for fish sperm, it is not necessary to bind the egg envelope (chorion). This may be one of the reasons why most fish sperm do not have an acrosome. In some fishes, the existence of an acrosin-like protein has been reported but its function still remains unknown.

After the sperm have attached to the oocyte plasma membrane, the fertilization wave moves through the cortex of the egg from the site of sperm attachment to the opposite site, the same as in the mammalian egg. Following the Ca^{2+} wave the contents of cortical granules are released to the space between oocyte plasma membrane and egg envelope (perivitelline space), and the fertilization envelope elevation occurs. After the cortical reaction, additional sperm cannot reach the oocyte plasma membrane.

Two-phase block to Polyspermy

Since Fol (1877) described his observation of fertilization including perivitelline space formation, fertilization envelope elevation, and a polyspermy block using sea urchin and starfish eggs for the first time, many investigators have tried to identify the molecular mechanisms of the polyspermy block.

In the sea urchin egg, within 30 seconds after the first sperm attachment to the oocyte plasma membrane, the egg envelope begins to elevate at the sperm attachment site. This envelope elevation is due to the release of cortical granule material in an exocytotic wave, triggered by the fertilization wave occurring from the sperm attached site. Within about one minute, the entire egg envelope is separated from the oocyte plasma membrane by a perivitelline space filled with the materials from the cortical granules. The elevated egg envelope, called the fertilization envelope, is thickened, hardened, and loses its affinity to bind sperm. These changes are caused by the interaction between the egg envelope and the materials secreted from the cortical granules (Fol 1877, Just 1919, Epel et al., 1977).

In 1952, Rothschild and Swann observed that the prevention of polyspermy is complete within one minute after sperm penetration and before the hardening of the fertilization envelope itself. Then Rothschild and Swann elaborated their hypothesis to a two-phase block to polyspermy using sea urchin fertilization model. According to their model, in the first 1~2 seconds after the first sperm attaches to the oocyte plasma membrane, certain invisible changes spread in the surface of the oocyte rendering it less permeable to other spermatozoa (rapid partial block phase). The second phase is slower and corresponds to changes visible in the cortex: after completion the egg surface is completely impermeable to spermatozoa (the full block phase). This hypothesis has been widely cited to explain the entire process of block to polyspermy.

The mechanisms of polyspermy block in the fish fertilization

The mechanism of the polyspermy block in fish eggs consist of at least four steps; (1) a polyspermy block related to changes in the structure of the micropyle to reduce the number of sperm reaching the oocyte plasma membrane, (2) formation of a fertilization cone derived from the sperm and oocyte plasma membranes to plug the inner opening of the micropyle (3) interaction between released cortical granule lectins and unfertilized supernumerary sperm in the micropylar vestibule, and (4) modification of the egg envelope by the contents released from the cortical granules (zona reaction including egg envelope hardening). This review will be focused on the events occurring in the micropylar region during the fertilization process related to the mechanisms of polyspermy block in the fish egg.

Step 1. Polyspermy block related to the structure of micropyle: The micropyle

In the fish egg there is the unique structural pathway for sperm to enter from the surface of the fibril layer of the oocyte to reach the oocyte plasma membrane, the micropyle. The micropyle is a funnel-shaped structure that consists of a vestibule and a micropylar canal (Ginzburg 1963, Kudo 1980, 1982, Laale 1980, Kobayashi and Yamamoto 1981, Hart and Donovan 1983). In the Chum salmon egg, the micropylar apparatus consists of a funnel-shaped vestibule from the bottom of which extends the micropylar canal, about 5 μm in diameter. The canal traverses the entire inner layer of egg envelope (zona pellucida interna of the egg envelope), about 30 μm in thickness. At its inner end, the diameter of the micropylar canal is the same as the width of the sperm head, about 2 μm. The lower portion of the canal is occupied by a conspicuous outgrowth of the ooplasmic surface, 3-10 μm in length (Kobayashi and Yamamoto, 1981). In most species, the base of micropylar canal is only wide enough for the passage of one sperm (Ginzburg 1972, Kuchow and Scott 1977, Brummett and Dumont 1979, Gilkey 1981, Iwamatu and Ohta 1981, Hart and Donovan 1983, Kudo et al., 1994, Leiher et al 1995), however, the micropyle in the carp egg is wide enough to admit several sperm at once (Kudo 1980). In most fish there is a single micropyle at the animal pole region, while in sturgeon and paddlefish there are several micropyles at the animal pole region (Ginzburg 1972, Cherr and Clark 1982, Linhart and Kudo 1997). During oogenesis the position of the micropyles(s) and its structure are determined by the micropylar cell or plug cell differentiated from the granulosa cells (Eigenmann 1890, Laale, 1980, Riehl 1978). It is obvious that the function of the micropyle during fish fertilization is to restrict the number of sperm to one or several from the numerous sperm trying to enter and attach the oocyte plasma membrane. In 1961, Sakai et al. reported that in Oryzias latipes, eggs with the envelope removed become polyspermic. This observation indicated the importance of the micropyle and egg envelope to the polyspermy block. Following the first sperm attachment to the oocyte plasma membrane through the micropyle, the egg surface responds to this stimuli and the Ca^{2+} wave moves along the egg cortex from the sperm entry site to the opposite side. Following this Ca^{2+} wave, the contents of cortical granule are released (cortical reaction) into the space between oocyte plasma membrane and egg envelope (perivitelline space formation).

The lack of the electrical polyspermy block

As described above, the process to prevent the supernumerary sperm into the oocyte consist of two different time lagged steps, 1) the rapid partial block phase, and 2) the full block phase. In some fish it seems that the fertilization wave may not be caused by the rapid partial block phase. In 1980, Nuccitelli et al. reported that the fertilization potential of Oryzias latipes has only a small positive going phase and voltage clamping the egg membrane at potentials between –80 and +48 mV does not prevent fertilization. In trout eggs, Ginzburg found that the trout egg remains fertile for a period of time after insemination in water (40 seconds at 3 ^{o}C and 8.9 ^{o}C), and then after 60 seconds fertility decreases sharply and is lost completely within 110-120 seconds. A cytological investigation indicated that the cortical secretion begins only after 60 seconds in water, i.e., 30-40 seconds after the activation wave appears. This means that the trout egg retains its original fertilization potential until the contents of the cortical granules begin to discharge into the perivitelline space (Ginzburg, 1963a,b, 1972, Kusa, 1950, Devillers et al., 1954, Zotin, 1954). It is not clear whether all fish lack an electrical polyspermy block; other species without an electrical block are the salamander (Charbonneau et al 1983) and some mammals (Miyazaki and Igusa, 1981, 1982, Igusa et al., 1983, Jaffe et al., 1983, McCulloh et a.,

1983, Braden 1954). The investigations in fish suggest that the function of the narrow channel for sperm entry, the micropyle, the interaction between the cortical granule contents and sperm during the cortical reaction, and the modification of the egg envelope by cortical granule contents are essential for the block to polyspermy.

Step 2. Formation of the fertilization cone: Fertilization cone

In 1981 Kobayashi et al. reported that in the Chum salmon egg the first contact of the ooplasmic surface with a fertilizing sperm occurred at the apex of the outgrowth in the micropylar canal. The surface of the outgrowth undergoes changes immediately after penetration by a fertilizing spermatozoon that prevents supernumerary spermatozoa from penetrating the ooplasm. Similar outgrowths from the egg ooplasm into the micropylar canal were observed in the sturgeon egg (Ginzburg 1959) and in the carp egg (Kudo 1980). In the *Fundlus heteroclitus* egg, a short cytoplasmic projection at the animal pole of the unfertilized egg was observed by Brummet and Dumont (1979) and sperm-oocyte plasma membrane fusion occurred at the apex of the projection inserted into the micropylar canal. In the zebrafish (*Brachydanio*) egg, a similar small projection in the micropyle was observed, while in the case of the medaka egg, a similar structure as the outgrowth in chum salmon or as a small projection in *Fundrus* and carp was not observed in the micropylar canal before or after the gamete fusion (Iwamatsu and Ohta 1978, 1981). This outgrowth and /or small projection from the egg cytoplasm into the micropylar canal has been termed the "fertilization cone" (Kudo 1980, Kobayashi and Yamamoto 1981, Hart and Donovan, 1983, Wolenski and Hart, 1987). It has also been reported that the fertilization cone is an actomyosin complex of the fused sperm and oocyte plasma membranes on (Gilkey et al., 1978, Hart et al., 1992, Abraham et al., 1993a,b) located between the ooplasm and the egg envelope to prevent a huge number of sperm access to the perivitelline space. It is obvious that the function of the fertilization cone in the block to polyspermy is to plug the inner opening of micropylar vestibule to block the supernumerary sperm from attaching and/or to penetrating into the space between egg envelope and oocyte plasma membrane.

Step 3. The interaction between released cortical granule lectins and supernumerary sperm in the micropylar vestibule

The cortical granules are specialized Golgi-derived secretory granules located just below the plasma membrane of mature unfertilized egg. As described above, after the first (fertilizing) sperm attaches to the oocyte plasma membrane, the fertilization wave moves through the cortex of the oocyte from the site of sperm attachment to the opposite side of the oocyte. Following this fertilization wave, the contents of cortical granule are released into the perivitelline space. It is known that cortical granules contents contribute to the block to polyspermy. In this section, the function of the cortical granule lectin in the polyspermy block during fish egg fertilization will be discussed. The function of other contents' contribution to the polyspermy block will be discussed in Step 4.

Egg Lectin

Since the first discovery of the contribution of an egg lectin in the polyspermy block in the anuran amphibian *Xenopus laevis* (Wyrick et al., 1974), our laboratory has focused on the

function of fish egg lectin during fertilization. Many researchers reported the existence of lectin in the fish egg, but its function was unknown. We chose the Chinook salmon as a model system to study the function of the egg lectin using biochemical and molecular biological methods because of its advantage in obtaining large amounts of biological materials and its economic importance in California. The eggs and milt were collected at the Nimbus State Hatchery (Department of Fish & Game, Nimbus, California) and transferred immediately to the lab in nearby Davis, California, at 4 ^{o}C. The egg lectin was purified with rhamnose-coupled affinity column chromatography and HPLC with ion exchange column and/or C4 reverse phase column chromatography. The salmon egg lectin (SEL) was found to consist of three subunits, SEL24K, SEL26Ka, and SEL26Kb, named by their molecular weights determined by SDS-PAGE. Each subunit of SEL was characterized using biochemical methods and cloned from a Chinook salmon ovary cDNA library (Murata et al., unpublished data).

Sperm agglutination and sperm immobilization activity of SEL24K

Sperm agglutination activity was measured by mixing 1/100 diluted milt and purified subunits of SEL that had been dialyzed against 10mM HEPES buffer pH 7.5. The sperm mixed with SEL24K agglutinated to each other immediately and this activity was inhibited by adding EDTA (10 mM) but not excess rhamnose. The inhibitory effect of sperm agglutination by EDTA was blocked by adding excess Ca^{2+} or Mg^{2+}. Sperm motility was also examined, and we found that SEL24K has sperm immobilizing activity, while SEL26K did not possess any sperm immobilizing activity. The sperm immobilizing activity by SEL24K was not inhibited by excess rhamnose. Therefore, SEL24K has three different activities: 1) carbohydrate binding activity, 2) sperm agglutination activity, and 3) sperm immobilizing activity. From our results, SEL24K should have at least two different domains, one is carbohydrate-binding domain and another is sperm binding domain (Murata et al., unpublished data).

The localization of the SEL in unfertilized and inseminated eggs

The localization of SEL was determined for the unfertilized and fertilized egg using immunohistochemical and immunocytochemical methods. In unfertilized eggs SEL was located not only in the large cortical granules but also in the small cortical granules just below the micropylar canal (Kobayashi et al., unpublished observations). The localization of the SEL ligand was also identified using purified SEL labeled with green fluorescence as a probe, and the signal was detected at just outside of the fibril layer of the outer layer of the vitelline envelope. Within one minute after insemination, SEL was located in the perivitelline space, the edge of the inner layer of the vitelline envelope, and in the micropylar vestibule in which a large number of sperm were observed (Kobayashi et al., unpublished observations). This data was supported by the detection of SEL in perivitelline fluid analyzed by Western blot. Two minutes after insemination the signal was still detected in the micropylar vestibule while the supernumerary sperm had disappeared from the micropylar aperture. Judging from these data, SEL was released from the cortical granules triggered by the fertilization wave into the perivitelline space, and filled the perivitelline space and micropylar vestibule. At this point SEL interacts with sperm to agglutinate and immobilize them, to push out the excess sperm from the micropylar vestibule as a fertilization plug, and to modify the fibril layer to hide the groove to the micropyle at the surface of fibril layer. The result is that sperm cannot find the micropyle in the fertilized egg. I

propose that SEL is the main molecule to contribute to the polyspermy block during the period from the beginning of the cortical reaction to the completion of the modification of egg envelope including egg envelope hardening. We have cloned medaka egg lectin subunits from a medaka ovary cDNA library (Murata et al., unpublished data).

In 1961 and 1972, Ginzburg reported the existence of a substance having sperm agglutination activity and sperm immobilizing activity in the perivitelline fluid of trout eggs. A similar observation was done in Chum salmon eggs by Janna and Yamamoto (1984) and in medaka eggs (Iwamatsu et al., 1997). These observations are also support my hypothesis. These functionally essential molecules may be conserved in many species. In 1980, in carp fertilized eggs, Kudo reported that in the perivitelline space near the canal, a few spermatozoa were frequently found and at about 6 seconds after immersion in fresh water to activate the eggs, agglutination of spermatozoa was usually observed in or near the micropylar vestibule. This observation suggested that in carp eggs it was possible that more than one sperm could enter into the inside of oocyte through micropyle but materials like SEL from cortical granules trapped them so that they could not penetrate the oocyte. This observation also strongly supported to our hypothesis.

Fertilization plug

Many investigators have observed that the slow burst of the perivitelline fluid through micropylar canal pushes excess sperm out of the micropyle and often forms a ball-like plug called the "fertilization plug" at the outside of the micropylar vestibule, composed of a colloidal substance originating from the cortical granules (Yamamoto 1952a,b, 1953, Sakai 1961, Kobayashi and Yamamoto 1981, Hart and Donovan 1983, Kudo and Sato 1985, Iwamatu et al., 1991, 1997). As described above, the fertilization plug is different from the fertilization cone. The fertilization plug is formed after the micropylar vestibule is plugged by the fertilization cone. This colloidal substance may include the cortical granule lectin to agglutinate and immobilize the excess sperm in the micropylar vestibule.

Step 4. Modification of the egg envelope by cortical granules contents (zona reaction including egg envelope hardening): The mechanisms of chorion hardening

During the fertilization process in fish, following gamete fusion, the morphological and biochemical changes in the extracellular matrix, the egg envelope (vitelline envelope, chorion) to the fertilization envelope, is the most dynamic transformation. According to the review of Yamamoto T., (1961), the first description of the characteristic differences between unfertilized egg envelopes and activated egg envelopes using *Onchorhynchus keta* was done by Aoki (1941). In 1958, Zotin found a hardening enzyme in the perivitelline space of salmon eggs that is secreted from the cortical layer but not from cortical alveoli (granules). The hardening process by this hardening enzyme is blocked by oxygen deficiency. In 1960, Ohtsuka described that the hardening is due to an oxidation of the SH-groups and aldehyde produced by oxidation of alpha-glycol groups. In medaka, the egg envelope consists of three major subunits, ZI-1 (76 kDa), -2 (74kDa) and -3 (49kDa), and their origin is the liver of spawning females but not the ovary (Murata et al., 1991, 1994, 1995, 1997). In 1991, Masuda et al. demonstrated that the solubility of the proteins in 1N NaOH decreases to 20% of that of proteins of unhardened egg envelopes 60 min after ionophore activation of eggs. During the early hardening process, ZI-1 and -2 undergo

limited hydrolyzed to produce 58-61 kDa proteins which are then polymerized with ZI-3 to form insoluble higher molecule weight complexes (135 kDa) (Masuda et al., 1991, 1992, Iuchi et al., 1995).

In this review, the function of two different enzymes involved in the chorion hardening will be discussed.

Alveolin

In 2000, Shibata et al. discovered that a metalloproteinase in the cortical granules triggered chorion hardening during the cortical reaction in the medaka egg. They purified it as a 21.5kDa protein from the exudates of the cortical alveoli (granules) and obtained the cDNA sequence for this metalloproteinase. From the predicted amino acid sequence, this metalloproteinase was identified as a member of astasin metalloproteinase family and was named "alveolin" after its origin, the cortical alveoli. Alveolin was found to process ZI-1 and -2 to 61-62 kDa intermediates, which are then cross-linked with ZI-3 by transglutaminase within the egg envelope itself.

Transglutaminase

The formation of gultamyl-lysine isopeptide crosslinks was suggested to be a cause of chorion hardening in trout eggs by Hargenmier in 1976. In 1994, Lee et al. characterized peptides released from the medaka-hardened egg envelope by the partial proteolytic action of medaka hatching enzyme and identified peptides containing significant amounts of γ-Glu-ε-Lys. These two results strongly suggested that a transglutaminase participated in chorion hardening because γ-Glu-ε-Lys formation is caused by transglutaminase. This possibility was strongly supported by results using cadavarine derivatives, which are competitive inhibitors of isopeptide crosslink formation (Oppen-Berntsen et al, 1990). The localization of the transglutaminse was indicated to be not in the cortical alveoli (granules) but in the egg envelope itself (Ha., et al 1995). In 1997 and 1998, Ha et al. purified two different forms of transglutaminase from unfertilized rainbow trout egg envelopes and characterized them as egg envelope hardening enzymes. Transglutaminase has been characterized and cloned from the livers of sea bream and chum salmon. However, the egg envelope form of the enzyme is apparently structurally different from the liver form.

It is obvious that the mechanisms of chorion hardening are controlled by at least two different enzymes. During the cortical reaction alveolin is discharged from the cortical granules into the perivitelline space and binds to the egg envelope, hydrolyzes the major components of egg envelope, ZI-1 and -2 (in case of medaka egg). The transglutaminase coexisting in the egg envelope (chorion) then polymerizes hydrolyzed ZI-1 and -2 with ZI-3 by peptide crosslinking.

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