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Chorion hardening in medaka (*Oryzias latipes*) egg

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Abstract The envelope, chorion, of unfertilized eggs of medaka (*Oryzias latipes*) consists mainly of glycoprotein components, ZI-1,2 (74–76-kD) and ZI-3 (49-kD). During the chorion hardening after egg activation, all the components are polymerized by the action of transglutaminase responsible for γ-glutamyl-ε-lysine crosslink formation. Prior to the polymerization, ZI-1,2 are cleaved by an enzyme(s) released from egg to change into 58–62-kD components. We discuss the possibility that the limited degradation of ZI-1,2 promotes the polymerization of chorion components. Thus the chorion hardening reaction is catalyzed by a multiple enzyme system.

Introduction

The unfertilized egg of various animals is enveloped by a certain number of layers of proteinaceous extracellular matrix. The innermost layer, the vitelline membrane of sea urchin or amphibian egg, and the zona pellucida of mammalian egg are found to participate in a species-specific sperm-egg interaction at fertilization, and to change into a fertilization membrane to prevent polyspermy (Schmell *et al.*, 1983). The conversion of unfertilized egg envelope to fertilized egg envelope has been studied in sea urchin (Foerder and Shapiro, 1977; Hall, 1978; Kay and Shapiro, 1987; Battaglia and Shapiro, 1988), in *Xenopus laevis* (Grey *et al.*, 1974; Wolf *et al.*, 1976; Gerton and Hedrick, 1986; Lindsay and Hedrick, 1989; Lindsay *et al.*, 1992; Lindsay and Hedrick, 1995), and in mouse (Gulyas and Schmell, 1980; Schmell and Gulyas, 1980; Wassarman and Mortillo, 1991).

The unfertilized fish egg envelope, the chorion, does not seem to participate in the specific binding to sperm for initiation of egg activation, but it does play as a mechanical barrier to block polyspermy (Sakai, 1961; Hart, 1990). After fertilization or activation of the egg, the envelope changes into a tough, hard structure resistant to environmentally-caused mechanical, chemical, or enzymatic disruption. Recently, the formation and degradation of the chorion in medaka, *Oryzias latipes*, have been extensively studied (See the review of Yamagami *et al.*, 1992). The medaka egg chorion consists of two layers: a thin outer layer and a thick inner layer. The outer layer, a minor part of the chorion, seems inert during the embryonic development, while the inner layer occupying the majority of chorion is a relatively simple and ordered multilamellar structure, which serves mainly as a protector for the developing embryo. Morphologically, the inner layer changes into a compact structure after egg activation (Nakano, 1956; Masuda *et al.*, 1992). In addition, the inner layer of unfertilized medaka egg chorion is soluble in some denaturants such as sodium dodecylsulfate (SDS), guanidium hydrochloride (GuHCl), and urea. Therefore, it is a suitable material for analysis of its molecular structure and the molecular mechanism of chorion hardening. Chorion hardening in fish eggs is essentially homologous to formation of the fertilization membrane in eggs of other animals.

Chorion hardening after egg activation

To understand the mechanism of chorion hardening, post-activation changes of egg toughness were examined. These changes have been estimated by two methods: measurement of the force required to compress an egg at a definite distance (Suga, 1963) and measurement of the weight required to rupture an egg (Iwamatsu, 1969). Recently, Urabe (1993) developed a new apparatus for the estimation of toughness. This method was based on measurement of resonance wave of an egg to extraneously-added mechanical vibration. Elasticity of the egg was calculated from the increase of frequency of the resonance wave. As shown in Fig. 1, elasticity of the medaka egg increased exponentially after fertilization and reached a maximum level at about 6 hr after fertilization at room temperature (blastula stage). The post-activation changes of elasticity of the medaka egg were essentially similar to those estimated by Suga (1963).

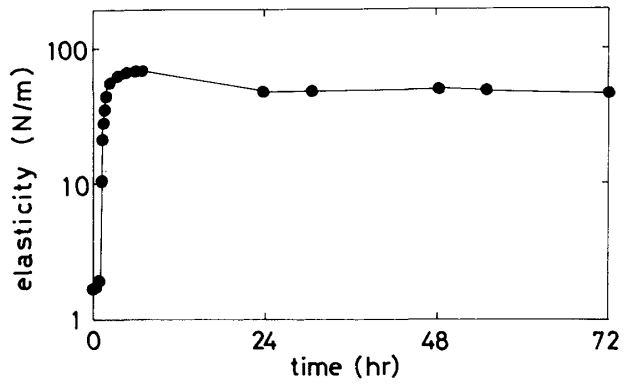

Fig. 1. Post-fertilization changes in elasticity of the medaka egg. The elasticity (N/m) is shown as a function of hours after insemination. From Urabe, 1993.

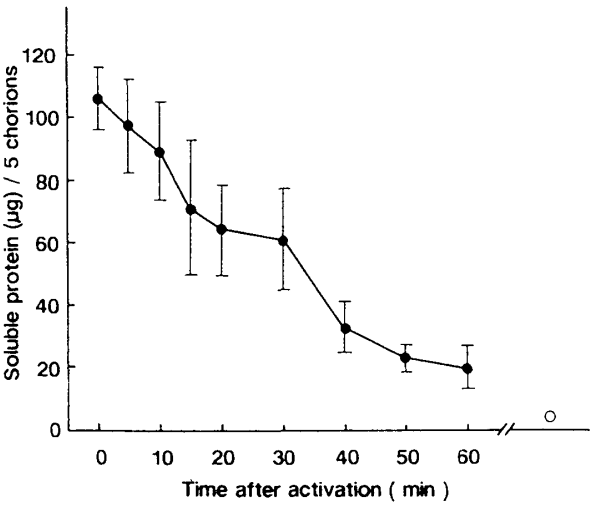

Fig. 2. Changes in solubility of chorion after egg activation. Unfertilized medaka eggs were incubated at 30°C in Yamamoto's physiological saline containing ionophore A23187 (20 μM). Chorions isolated after activation were dissolved by incubation in 1N NaOH at 30°C for 30 min. The open circle shows the soluble protein in chorions of blastulae. From Masuda *et al.*, 1991.

Zotin (1958) reported a way to estimate directly the hardness of chorion isolated from eggs of the salmonid fishes. This method involved measurement of weight required to break down a chorion sheet. The increase of chorion hardness thus measured paralleled the post-activation increase of egg toughness. Although this method does not show us a well-defined physical characteristic such as elasticity, the result is evidence that increased egg toughness after activation is essentially dependent on increased chorion hardness. There is yet no direct measurement of the physical character of medaka egg chorion itself.

Recently, chorion rigidity or hardness in medaka egg was deduced from another viewpoint. Masuda *et al.* (1991) examined changes of solubility of chorion in 1N NaOH. It is conceivable that changes in chorion solubility in some denaturants depend upon changes of physico-chemical structure of the chorion such as formation of certain covalent crosslinks in it, modification of non-covalent interaction between its constituent proteins, and the resultant compaction of the chorion. As shown in Fig. 2, solubility was decreased with chorion hardening after egg activation by ionophore A23187. SDS (1–6%) or urea (8M) could also dissolve the soft chorion of an unfertilized egg but not hardened chorion. Therefore, deceased chorion solubility in NaOH, SDS or urea is regarded as an index of post-activation increase of chorion hardness.

Changes of protein components of chorion after egg activation

The inner layer of the medaka oocyte chorion consists mainly of protein components, ZI-1,2 (see footnote) and ZI-3 (Hamazaki *et al.*, 1985). The origin and some biochemical characteristics of these constituents have been studied extensively (Hamazaki *et al.*, 1987a,b, 1989; Murata *et al.*, 1991, 1993, 1994). Recently, Murata *et al.* (1995) cloned cDNA for the precursor protein of ZI-3 and deduced its amino acid sequence. ZI-3 is homologous to a zona pellucida protein, ZP3, of mammalian eggs.

We examined changes of chorion protein components after egg activation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3). Unfertilized egg chorions consisted mainly of ZI-1,2 (74–86-kD) and ZI-3 (49-kD), essentially similar to those of oocyte. After egg activation, ZI-1,2 and ZI-3 decreased in amount, 58–62-kD and 110–130-kD components appeared, and finally, no protein bands became detectable on SDS-PAGE. Thus, one of the characteristic events occurring after egg activation was the appearance of several high-molecular-weight components in the chorion.

The high-molecular-weight components of oocyte chorion were first identified as two distinct proteins, ZI-1 and ZI-2 (Hamazaki *et al.*, 1984). Further studies suggested that these proteins were nearly identical in molecular weight (Hamazaki *et al.*, 1984), amino acid composition (Murata *et al.*, 1993; Sugiyama *et al.*, unpublished), and the behavior in their limited degradation after egg activation (Masuda *et al.*, 1991). Therefore, ZI-1 and ZI-2 were considered to form a group of proteins, and renamed ZI-1,2 as a whole.

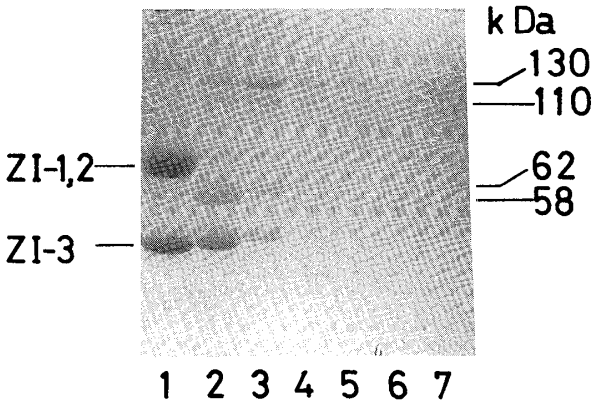

Fig. 3. Changes in SDS-PAGE patterns of chorion proteins after egg activation (8% gel). Unfertilized medaka eggs were incubated at 25°C in Yamamoto's physiological saline containing ionophore A23187 (40 μM). Lanes 1 to 7 represent 0, 15, 30, 45, 60, 75, and 90 min after egg activation, respectively.

Egg envelope transglutaminase

Previous studies concerning the mechanism of chorion hardening in fish egg have suggested participation of various reactions or factors such as environmental Ca^{2+} (Kusa, 1949a,b; Ohtsuka, 1957; Yamamoto, 1957), pH (Iwamatsu, 1969), oxygen (Zotin, 1958), cortical alveolar materials and mucopolysaccharides (Nakano, 1956), and oxidation of the sulfhydryl group in chorion protein (Ohtsuka, 1957, 1960). In chorion hardening of salmonid fish egg, Zotin (1958) has suggested occurrence of the hardening enzyme released from unfertilized eggs by cortical reaction after activation.

We studied the mechanism of appearance of high-molecular-weight protein components in the chorion during hardening. When chorions isolated from unfertilized eggs were incubated with Ca^{2+}, solubility of the chorions in 1N NaOH or SDS (1–6%) was decreased (Masuda *et al.*, 1991), showing that factors required for the chorion hardening reaction are present in the chorion itself. This phenomenon was tentatively named "*in vitro* Ca^{2+} hardening" to distinguish it from the chorion hardening occurring in an activated egg, "*in situ* hardening". During the *in vitro* Ca^{2+} hardening, the amounts of ZI-1,2 and ZI-3 decreased, components having the molecular weight higher than 148K appeared, and finally, all components became undetectable on SDS-PAGE, as shown in Fig. 4 (Masuda *et al.*, 1992). The 148-kD component was found to be formed by covalent binding of ZI-1,2 and ZI-3 because it was stainable with both anti-ZI-1,2 antibody and anti-ZI-3 antibody. Therefore, the *in vitro* Ca^{2+} hardening is a good model for studying the formation of high-molecular-weight components during chorion hardening. *in vitro* Ca^{2+} hardening was heat-labile, optimal at pH 5–6, and inhibited by iodoacetamaide (IAA; 10 mM), ethylenediaminetetraacetic acid (EDTA; 5 mM), and monodansylcadaverine hydrochloride (2 mM), a competitive inhibitor of transglutaminase responsible for γ-glutamyl-ε-lysine (γ-Glu-ε-Lys) crosslink formation between polypeptides.

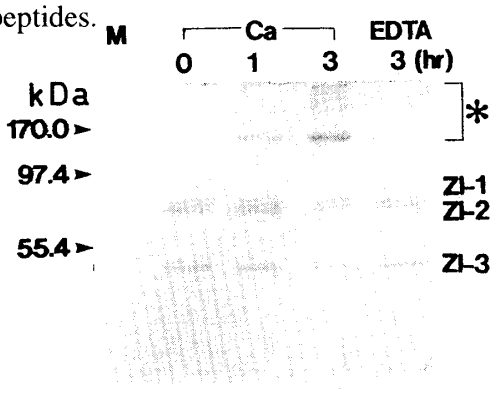

Fig. 4. Changes in SDS-PAGE patterns of chorion proteins during *in vitro* Ca^{2+} hardening (8% gel). Homogenates of chorions isolated from unfertilized medaka eggs were incubated with isotonic saline containing 5 mM $CaCl_2$ (Ca) at 30°C for 0, 1 or 3 hrs. As a control, the chorion homogenate was incubated for 3 hrs in the isotonic saline containing 5 mM EDTA. * indicates a 148-kD protein and proteins having a molecular weight higher than 148K. M shows a pattern of marker proteins. From Masuda *et al.*, 1992.

To understand covalent crosslinks formed between chorion constituents during hardening, we made a preliminary estimate of the content of γ-Glu-ε-Lys isopeptide in the medaka chorion: it was 32.8 nmoles/mg of dry chorion in unfertilized eggs, while it was 63.6 nmoles/mg of dry chorion in blastulae (unpublished data). Weight of the chorion was not significantly changed after hardening. The γ-Glu-ε-Lys content in the unfertilized egg chorion is probably overestimated because it is possible that *in vitro* Ca^{2+} hardening had been partially induced during the preparation of the sample. On the other hand, the γ-Glu-ε-Lys content in blastula chorion is probably underestimated: our preliminary study did not verify complete digestion of the hardened chorion by the used proteases, i.e., pronase E, leucine aminopeptidase, carboxypeptidase Y and prolidase. Although the present method of estimation is limited and more precise values should be determined in the future, it is evident that there is a post-activation formation of γ-Glu-ε-Lys crosslinks between chorion constituent proteins in medaka as well as in rainbow trout egg chorion (Hagenmaier *et al.*, 1976; Iuchi *et al.*, 1994).

Occurrence of crosslinks other than γ-Glu-ε-Lys isopeptide was well known in some fibrous, elastic or hard proteins. Tyrosine-tyrosine (Tyr-Tyr) bridges have been found in the chorion or vitelline membrane in the laid eggs of *Drosophila melanogaster* (Petri *et al.*, 1976) and in the fertilization membrane of sea urchin embryos (Foerder and Shapiro, 1977; Nomura *et al.*, 1990). In sea urchin eggs, peroxidase (ovoperoxidase), an enzyme responsible for Tyr-Tyr bridge formation, was identified in cortical granules and was found to be released from them on activation. Histochemical and biochemical studies using synthetic substrate or inhibitors for ovoperoxidase suggested the presence of this enzyme in mouse egg cortical granules and its participation in zona hardening (Gulyas and Schmell, 1980; Schmell and Gulyas, 1980). In unfertilized egg chorions of medaka, di- or tri-tyrosine crosslinks were negligible in amount and no significant increase was found in the chorions after fertilization (Nomura, personal communication). In addition, a high concentration (100 mM) of aminotriazole, an ovoperoxidase inhibitor, did not inhibit either the *in situ* or *in vitro* Ca^{2+} hardening of medaka egg chorion (Masuda *et al.*, 1991, 1992).

As suggested by Ohtsuka (1960), disulfide bridge formation between chorion components seems to play an important role in chorion hardening. Cysteine and/or cystine content in unhardened or hardened chorions should be examined in the future. In addition, we have not yet any information on occurrence of other covalent bridges such as desmosine in elastin molecule or lysinonorleusine in collagen molecule.

Recently, the structure of hardened chorion in blastula was examined using hatching enzyme, chorion-digesting protease (Lee *et al.*, 1994). The hatching enzyme of medaka embryo consists of two enzymes: high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE). HCE swells the inner layer of intact hardened chorion by its partial proteolytic action, and while LCE cannot digest the intact chorion, it can completely digest the swollen chorion (Yasumasu *et al.*, 1989a,b,c). We characterized several peptides released from the hardened chorion by the partial proteolytic action of HCE. Among them there were the peptides containing significant amounts of γ-Glu-ε-Lys and proline in the form of peptides consisting of Pro-x-y repeats (Lee *et al.*, 1994). Failure of LCE to digest the intact hardened chorion is probably ascribable to occurrence of such a unique γ-Glu-ε-Lys-rich, proline-rich structure in the chorion. In fact, LCE can easily digest intact unhardened chorion of unfertilized egg. The results show that a unique, LCE-resistant structure is formed in the hardening chorions.

Because transglutaminase (TGase) responsible for γ-Glu-ε-Lys crosslink formation is considered to participate in chorion hardening in fish eggs (Hagenmaier *et al.*, 1976; Oppen-Berntsen *et al.*, 1990; Masuda *et al.*,1991), we assayed TGase activity in unfertilized egg according to the method of Lorand and Gotoh (1970). TGase, a Ca^{2+}-dependent SH-enzyme, was detectable in unfertilized medaka egg, and localized exclusively in chorion fraction (Fig. 5). This is the first demonstration of chorion TGase in medaka egg (Ha *et al.*, 1995). Thus the unfertilized egg chorion is a unique structure: an enzyme, TGase, and its substrate, chorion, coexist.

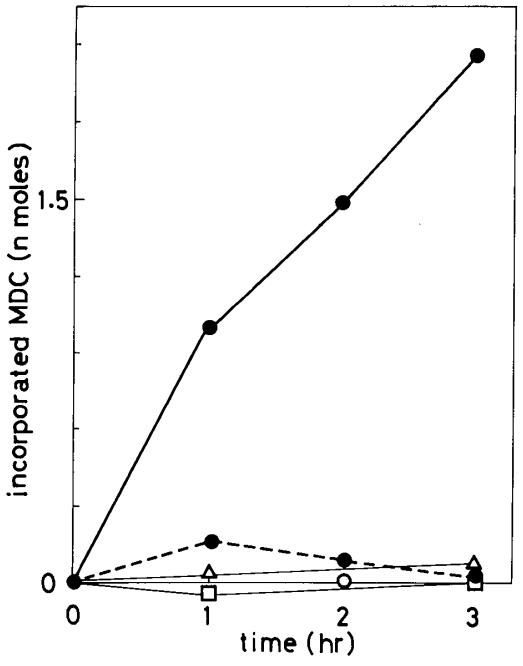

Fig. 5. Time course of TGase activity of unfertilized medaka eggs. After unfertilized eggs in isotonic saline were cut with small scissors, chorions were separated from the remnants (egg fraction). Homogenate of the chorions in isotonic saline (●—●) or the egg fraction (●---●) was used as enzyme sample. The reaction mixture consisted of 0.5 mM monodansylcadaverine (MDC), 0.2% N,N-dimethylcasein, 5 mM $CaCl_2$, 5 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 7.2) and enzyme sample equivalent to 0.45 eggs. TGase activity was assayed at 30°C and expressed by n moles of MDC incorporated into casein. The chorion TGase was inhibited by EDTA (10 mM; △), IAA (10 mM: □), or absence of Ca^{2+} (○).

Chorion hardening reaction as a reaction catalyzed by a multiple enzyme system

Here we again observed precisely the changes of constituent proteins of chorion during the *in situ* hardening, and noticed the appearance of 58–62-kD components. As shown in Fig. 3, the appearance of the 58–62-kD components in the hardening chorion seemed to be accompanied not by a decrease of ZI-3 but by a decrease of ZI-1,2. This suggested that a limited degradation of ZI-1,2 (74–76-kD) into 58–62-kD components occurs during chorion hardening (Masuda *et al.*, 1991). When unfertilized egg was activated by ionophore A23187 in the presence of IAA (10 mM) to inhibit the chorion TGase, the chorion hardening was seriously inhibited. SDS-PAGE analysis failed to detect high-molecular-weight components such as 110–130K or 148K, and in addition, the amount of ZI-3 was not significantly changed during the incubation. However, ZI-1,2 decreased in amount and 58–62-kD components appeared. Therefore, these findings suggest that a limited degradation of ZI-1,2 into 58–62-kD components occurs during the chorion hardening and is not inhibited by IAA. Because ZI-1,2 are glycoproteins (Hamazaki, 1987), the limited hydrolysis may be performed either by protease or by glycosidase.

We have some information on the limited degradation of chorion components. When an unfertilized egg of rainbow trout (*Oncorhynchus mykiss*) was activated by immersion in water, toughness of the egg and hardness of the chorion were increased as in the medaka egg (*in situ* hardening). After the chorion begins to harden, 49-kD, 56-kD, and 65-kD constituent proteins of the unfertilized egg chorion decreased in amount, 113-kD, 160–170-kD, and higher than 250-kD proteins appeared, and finally, any protein bands became undetectable on SDS-PAGE. Formation of high-molecular-weight components is responsible for the chorion hardening as in the medaka egg. When incubated with Ca^{2+}, the chorion isolated from unfertilized egg hardened by the formation of high-molecular-weight components as a result of the action of TGase localized exclusively in the chorion (*in vitro* Ca^{2+} hardening). However, *in vitro* Ca^{2+} hardening was much slower than that of the *in situ* hardening. Therefore, we searched for an *in vitro* Ca^{2+} hardening-promoting activity in the egg.

We dissected unfertilized eggs in isotonic saline with small scissors and suspended them by gentle pipetting. After the chorions were removed, the suspension was centrifuged at 850 x *g* for 15 min. The precipitate probably contained egg components such as cortical alveoli. When the homogenate of the precipitate was added to the reaction mixture of the *in vitro* Ca^{2+} hardening system, the hardening reaction measured by solubility of chorion in 8M urea was promoted. The *in vitro* Ca^{2+} hardening-promoting activity (HPA) did not exhibit TGase activity, and it could not significantly activate TGase activity in the chorion. In addition, we found evidence that HPA contains a factor(s) responsible for the limited degradation of a 49-kD component of chorion to 44-kD component. Therefore, it is possible that HPA promotes chorion hardening not by activating TGase in the chorion, but by hydrolyzing the 49-kD component of the chorion to the 44-kD component. The action of HPA on the chorion hardening could not be inhibited by a relatively high concentration (200 μg/ml) of various protease inhibitors such as leupeptin, elastinal, chymostatin, antipain, phosphorhamidon, or E_{64}, while it was inhibited by EDTA (20 mM). In addition, EDTA inhibited the HPA-dependent limited degradation of the 49-kD component to the 44-kD component. HPA is heat-labile, and it is considered to be an EDTA-sensitive enzyme(s). As in the case of rainbow trout egg, we have evidence that HPA activity exists in the cortical alveolar fraction of the unfertilized medaka eggs.

Although many problems remain to be solved and many precise studies should be made in the future, we tentatively propose a working hypothesis for studies on chorion hardening in fish egg: when an unfertilized egg is activated, an enzyme(s) is released from the egg to cause limited degradation of a chorion component(s), and then, TGase localized in the chorion acts on the modified chorion to form efficiently γ-Glu-ε-Lys crosslinks between chorion components, resulting in chorion hardening.

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