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Evidence for Involvement of the Exudate Released from the Egg Cortex in the Change in Chorion Proteins at the Time of Egg Activation in *Oryzias latipes*

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ABSTRACT—In eggs of the medaka *Oryzias latipes*, we recently found that the changes in chorion proteins that occur upon fertilization could be induced *in vitro* by an egg exudate including cortical alveolar contents. In the present study, the time course of the changes in chorion proteins induced *in vitro* by egg exudate was examined by SDS-PAGE analysis and was found to be quite similar to that of *in vivo* chorion hardening at the time of fertilization. The activity of egg exudate was dose-dependent. Moreover, when the exudate was heated for 30 min at 60°C, it lost its activity. On the other hand, in heat-treated chorions, the intermediate proteins appeared following incubation with egg exudate, although chorion hardening did not occur. These results suggest the presence of a chorion protein-modifying enzyme(s) in the exudate released from the cortex during egg activation in *Oryzias latipes*.

INTRODUCTION

In oviparous species, the egg is enveloped by an extracellular coating. The egg envelope of fishes is generally called the chorion. Progressive biochemical and structural changes in the egg envelope occur following cortical reaction at the time of fertilization. It is currently believed that the contents of the cortical granules (vesicles), which are exocytozed from the egg cortex into the perivitelline space, are involved in transforming the egg envelope during egg activation (Schuel, 1978). In the sea urchin, change in the egg envelope is promoted by a cortical granule-derived ovoperoxidase which catalyzes the formation of dityrosine crosslinks between component proteins of the egg envelope (Foerder and Shapiro, 1977; Hall, 1978; Klebanoff *et al.*, 1979). In amphibian eggs, a cortical granule lectin is reportedly involved in the transformation of the egg envelope (Grey *et al.*, 1974; Wyrick *et al.*, 1974; Yoshizaki and Katagiri, 1984). In addition, proteases that partially hydrolyze the vitelline envelope glycoproteins are released from amphibian eggs at the time of fertilization and participate in the conversion of the vitelline envelope into the fertilization membrane (Lindsay and Hedrick, 1989). In fishes also, the mechanism of chorion hardening has been investigated, but still has not been clarified (Yamagami *et al.*, 1992). Although transglutaminase has been reported to participate in chorion hardening of fish eggs by forming glutamyl-lysine isopeptide crosslinks (Hagenmaijer *et al.*, 1976; Oppen-Berntsen *et al.*, 1990), the enzyme has not yet been isolated.

In the medaka *Oryzias latipes*, we recently found that changes in the chorion proteins could be induced *in vitro* by egg exudate containing cortical alveolar contents (Iwamatsu *et al.*, 1995) which had been exocytozed into the perivitelline space at the time of fertilization. The present report provides definite evidence that the substance(s) released from the egg cortex is involved in chorion hardening and suggests the existence of a chorion protein-modifying or chorion hardening enzyme(s) in egg exudate, the activity of which is heat-labile and dose-dependent.

MATERIALS AND METHODS

Preparation of chorions from unfertilized eggs

Within 2 hr after ovulation, unfertilized medaka eggs were removed from the ovarian lumen into saline (111.2 mM NaCl, 5.4 mM KCl, 1.1 mM $CaCl_2$, 0.6 mM $MgSO_4$, pH adjusted to 7.3 with M/2 $NaHCO_3$) by a routine procedure (Iwamatsu *et al.*, 1976). The chorions of unfertilized eggs were isolated by using small scissors to cut the vegetal pole side of the chorion and then using watch-maker forceps to squeeze the intact vitellus out through the large hole, as described elsewhere (Iwamatsu, 1983). Unhardened chorions were freshly prepared for each experiment and kept before use on ice (0–4°C) in chilled, calcium-free saline (128.3 mM NaCl, 27 mM KCl, 6.0 mM $NaHCO_3$, 0.2 mM EDTA, 4 mM HEPES-NaOH, pH 7.0) within a small Petri dish (diameter 35 mm). The saline used in the present experiments was previously filtered with a Millipore filter (pore size 0.20 μm, Advantec).

Collection of egg exudate

At the same time unhardened chorions were prepared, naked eggs were obtained as described above. The collection of egg exu-

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date from the naked eggs was performed according to the procedure of Iwamatsu *et al.* (1995). The volume of egg exudate was diluted to various concentrations before use by adding sterile saline.

Incubation of isolated chorions with egg exudate

Isolated chorions from unfertilized eggs were rinsed in saline and then put in a polystyrene tissue culture dish (sterile, 35×10 mm, Iwaki Glass Co., Chiba, Japan). After saline was removed by a micropipette, the chorions were mixed with undiluted or diluted solutions of egg exudate and incubated at 27°C. In an experiment on the heat-lability of egg exudate activity, freshly prepared egg exudate was heated in an Eppendorf tube for 30 min at 60°C in a water-bath just before use. In another experiment, isolated chorions were heat-treated before use in an Eppendorf tube for 30 min at 60°C with calcium-free saline.

Extraction and gel electrophoresis of chorion proteins

At the end of incubation, chorions were rinsed once in saline and transferred into a chilled Potter-Elvehjem homogenizer. After removal of the saline, protein extraction medium (5 M urea, 1.5 M thiourea, 10 mM pyrophosphate, 0.5% 2-mercaptoethanol, 0.1 mM TLCK) was added, and the contents immediately homogenized for 5 min (1,500 rpm) at 0–4°C. The homogenate was incubated in a water-bath at 60°C for 3 min to inactivate proteolytic enzymes and then immediately chilled on ice. The sample (2.5–3 mg proteins/ml) was frozen and stored at –20°C for later use. Each sample was mixed with an equal volume of a sample buffer and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide in separating gels which were prepared and run according to the procedure of Laemmli (1970). After electrophoresis, the gels were fixed with 10% acetic acid and 45% methanol for 1.5 hr, pretreated for 30 min with 0.1 M picric acid (neutralized with NaOH to pH 7), and stained with 0.05% Coomassie brilliant blue G250 in a mixture of 75 mM picric acid, 2.5% acetic acid and 11% methanol. The gels were destained with a mixture of 5% methanol and 10% acetic acid and rinsed in tap water using a Plus Shaker EP-1 (Taitec Co.).

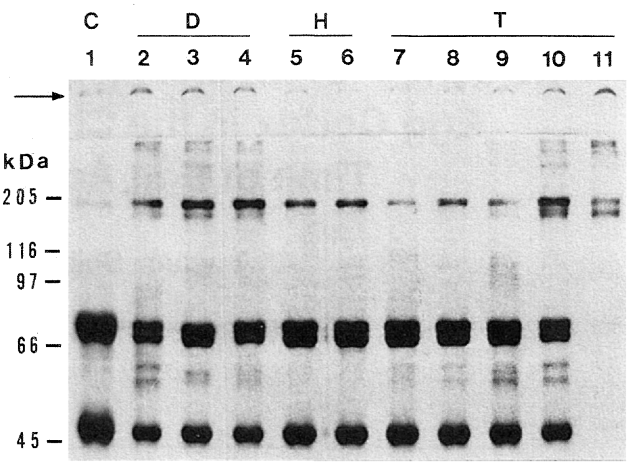

Fig. 1. The change in SDS-PAGE (10% separation gel) patterns of chorion proteins induced by egg exudate. (C) Chorion of unfertilized egg without treatment (lane 1). (D) Isolated chorions of unfertilized eggs were incubated in diluted (1, 1/2, 1/4) solutions of egg exudate (starting at 1/12.5 of the concentration in the perivitelline space) for 30 min at 27°C. Lane 2, undiluted; lane 3, 1/2 concentration; and lane 4, 1/4 concentration. (H) Isolated chorions were incubated for 30 min (lane 5) and 60 min (lane 6) 27°C in egg exudate which had been heated for 30 min at 60°C. (T) In order to examine the time course of the changes in chorion proteins induced by egg exudate, isolated chorions of unfertilized eggs were incubated at 27°C for 3 min (lane 7), 5 min (lane 8), 10 min (lane 9), 30 min (lane 10) and 60 min (lane 11) in egg exudate. The left margin of the gel has molecular mass standards. Arrow indicates proteins stuck in the wells of the stacking gel (5%). Each lane contains 15 μg of proteins.

RESULTS

The time course of the changes in chorion proteins induced by egg exudate

Three minutes after the incubation of isolated chorions in egg exudate commenced, faintly stained intermediate (62kDa and 61kDa) protein bands were already detected on SDS-PAGE gels (Fig. 1, lane 7). With 5 min of incubation, a 132kDa band appeared faintly (Fig. 1, lane 8). The 150kDa band became gradually more definite from 5 min to 30 min after the initiation of incubation. By 60 min after the initiation of incubation, the 77–73kDa, 62kDa, 61kDa and 49kDa proteins had mostly disappeared, while the 150kDa and 132kDa proteins were still detectable (Fig. 1, lane 11). The high molecular weight proteins, which were trapped in the wells of the stacking gel or on the top surface of the separation gel, gradually increased up to 60 min after the initiation of incubation.

Effect of heat-treated egg exudate on the change in chorion proteins

Isolated chorions were incubated at 27°C in a water-bath, in egg exudate which had previously been heated for 30 min at 60°C. When chorions were incubated for 30 min in the heated egg exudate, the 62kDa, 61kDa, 132kDa, 114kDa and higher molecular weight protein bands did not appear on SDS-PAGE gels (Fig. 1, lane 5). The staining of the 77–73kDa and 49kDa proteins remained unchanged, whereas the 150kDa band was more strongly stained than that of the untreated chorions (Fig. 1, lane 1). The proteins of the chorions incubated for 60 min showed the same electrophoretic pattern as those incubated for 30 min. The pattern was entirely different from that produced by non-heated egg exudate (Fig. 1, lanes 5 and 6 vs 10 and 11).

Effect of the diluted egg exudate on the change in chorion proteins

In order to investigate the dilution effect on the activity of egg exudate, the exudate (starting at 1/12.5 of the concentration in the perivitelline space) was diluted to 1, 1/2 and 1/4 of the original concentration and incubated with isolated chorions for 30 min (Fig. 1, lanes 2, 3 and 4). When the chorions were incubated in the undiluted egg exudate, the two bands at 77–73kDa and 49kDa were weakly stained with a concomitant slight decrease in the 150 and 132kDa on the gels by Coomassie blue, while the bands of intermediate proteins were heavily stained. The quantity of intermediate proteins and high molecular weight proteins, which

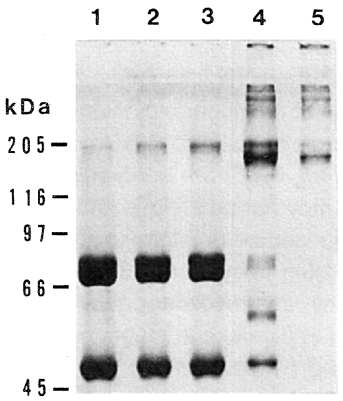

Fig. 2. The changes in component proteins of chorions incubated in egg exudate inserted so that it would act from the inner surfaces of isolated chorions. Isolated chorions of unfertilized eggs (lane 1) were incubated in saline alone for 30 min (lane 2) or 60 min (lane 3) at 27°C as controls. In lanes 4 and 5, egg exudate (1/4 of the concentration in the perivitelline space) was introduced into the insides of isolated chorions which were then incubated for 30 min (lane 4) or 60 min (lane 5) 27°C. The left margin of the gel has molecular mass standards. Each lane contains 15 μg of proteins.

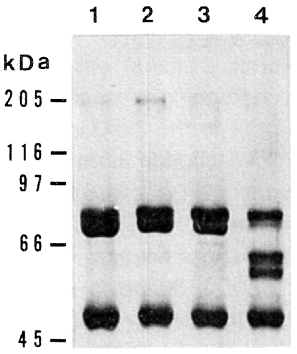

Fig. 3. The changes in component proteins of pre-heated chorions induced by egg exudate. Isolated chorions of unfertilized eggs (lane 1) were pre-heated for 30 min at 60°C (lane 3) and then incubated in egg exudate for 30 min at 27°C (lane 4). In lane 2, isolated chorions were incubated in saline alone for 30 min at 27°C as a control. The left margin of the gel has molecular mass standards. Each lane contains 15 μg of proteins.

were trapped in the wells of the stacking gel or on top of the separation gel, also gradually increased in proportion to the increase in the concentration of egg exudate.

Changes in chorion proteins induced by egg exudate that was introduced into the inside of the chorion

In the process of *in vivo* chorion hardening, egg exudate is released into the perivitelline space and exerts its action on the chorion from the inner side. In order to examine the effect of the direction of action, egg exudate (1/4 of the concentration in the perivitelline space) was introduced by a small bore pipette into the inside of isolated chorions through a large hole at the vegetal pole side of each. The preparations were then incubated for 30 min or 60 min at 27°C (Fig. 2, lanes 4 and 5). Thirty minutes after the initiation of incubation, the high molecular weight protein bands on the gels were strongly stained by Coomassie blue, while the 77–73kDa and 62kDa proteins were stained more faintly than the 61kDa and 49kDa protein bands. The change in the electrophoretic patterns of chorion proteins in this *in vitro* experiment duplicated that observed with *in situ* chorion hardening (Iwamatsu *et al.*, 1995). In order to verify this effect of egg exudate, isolated chorions were incubated in saline alone for 30 min or 60 min at 27°C (Fig. 2, lanes 2 and 3). The proteins of the incubated chorions formed the same electrophoretic pattern as those of non-incubated chorions.

The changes in component proteins of heated chorions induced by egg exudate

In order to ascertain whether an enzyme(s) pre-exists within the chorion, isolated chorions were heated for 30 min at 60°C (Fig. 3, lane 3) and then incubated in egg exudate for 30 min at 27°C (Fig. 3, lane 4). The egg exudate used in this experiment was part of the same egg exudate preparation (1/4 of the concentrations in the perivitelline space) used in the above experiment. Isolated chorions had lost their elasticity at the end of heat-treatment. In these chorions incubated with egg exudate, the 62kDa and 61kDa proteins on the gels were stained by Coomassie blue, although these bands did not appear in chorions incubated in the absence of egg exudate (Fig. 3, lane 3). In addition, the staining of the 77–73kDa protein was weaker. However, the high molecular weight proteins could not be detected, and chorion hardening did not occur.

DISCUSSION

We previously proposed that the change in chorion proteins (chorion hardening) takes place through enzymatic mechanisms at the time of fertilization, because the changes in chorion proteins that could be induced *in vitro* by incubation of unfertilized egg chorions in egg exudate were temperature- and pH-dependent (Iwamatsu *et al.*, 1995). Chorion hardening in the rainbow trout egg is also temperature- and pH-dependent (Iuchi *et al.*, 1991). In addition, the present study demonstrated that the activity of egg exudate to induce changes in chorion proteins was heat-labile and dose-dependent. These results strongly suggest the presence of an enzyme(s) in the egg exudate which induces the changes in chorion proteins during chorion hardening.

The time course of the *in vitro* changes in component proteins of isolated chorions induced by egg exudate was

very similar to that of the *in vivo* changes in chorion proteins at fertilization. However, a slight difference in the time course of disappearance of the 77–73kDa proteins and in the temporary appearance of high molecular weight proteins in the electrophoretic patterns was recognized between *in vitro* and *in vivo* hardening processes. The 77–73kDa protein disappears prior to the 62kDa, 61kDa and 49kDa proteins during *in vivo* chorion hardening (Iwamatsu *et al.*, 1995), whereas the 62kDa and 61kDa proteins disappear concurrently with the 77–73kDa and 49kDa proteins as *in vitro* chorion hardening proceeds. This electrophoretic pattern of chorion proteins during *in vitro* hardening is somewhat similar to that induced by artificial treatments with high Ca^{2+} concentrations (Masuda *et al.*, 1991; Iwamatsu *et al.*, 1995) or gum arabic (Iwamatsu *et al.*, 1995). However, this similarity dose not seem to indicate that Ca^{2+} causes chorion hardening, since no change in chorion proteins could be recognized following incubation in saline alone in the present study. The different electrophoretic pattern with *in vitro* treatment from that seen in natural hardening may be due to (1) reduced activity of the enzyme(s) caused by dilution of the egg exudate and (2) the enzyme(s) acting from the outside of the chorion. In the present *in vitro* experiments, egg exudate was diluted about 12.5 times based on the estimated volume of the pervitelline space. In fact, in another experiment in which the egg exudate was used at a higher concentration (1/4 of the concentration in the perivitelline space) and placed where it would act from the inside of the chorion, the changes in chorion proteins became more similar to those seen with *in vivo* chorion hardening.

Since the hardening process of chorions isolated from Ca^{2+}-ionophore-activated eggs could be interrupted by removal of Ca^{2+} from the medium and resumed upon readdition of Ca^{2+}, Masuda *et al.* (1991) suggested that the isolated egg chorions contain some 'hardening machinery' that functions with Ca^{2+}. It is possible that the chorion hardening enzyme(s) pre-exists within the chorion and acts in the presence of Ca^{2+} at physiological concentrations. In the present experiment, the change in chorion proteins was induced by egg exudate in saline that does not induce chorion hardening by itself. The heated egg exudate lost its activity. Moreover, heated chorions showed the appearance of intermediate proteins characteristic of natural chorion hardening, although no high molecular weight proteins appeared at the end of 30 min incubation in egg exudate. These experimental results reveal that the early step of chorion hardening is induced by a chorion protein-modifying enzyme(s) present in the egg exudate rather than in the chorion.

The chorion proteins associated with polymerization are considered to be heat-labile, judging from the observation that no polymerization of proteins takes place in heated chorions treated with egg exudate. It is plausible that heat-treatment may give rise to conformational changes in chorion proteins which prevent polymerization by enzymatic action, since the hardening of isolated chorions depends upon egg exudate. The results of our study, however, do not preclude the possibility that a enzyme or co-enzyme participating in polymerization of chorion proteins pre-exists within the chorion.

The present results also suggest that the processes of polymerization of chorion proteins and transformation of the 77–73kDa protein to the 62kDa and 61kDa proteins (Iwamatsu *et al.*, 1995) are separable during chorion hardening. There may not be a close and direct relationship between the appearance of intermediate proteins and chorion hardening judging from a previous report (Iwamatsu *et al.*, 1995) that the intermediate proteins appear before the chorion begin to increase in toughness. The appearance of the 62kDa and 61kDa proteins is probably due to partial hydrolysis of chorion proteins (Masuda *et al.*, 1991; Iwamatsu *et al.*, 1995). Hydrolysis of component proteins of the egg envelope by protease which is released from the activated egg has been reported in the sea urchin (Vacquier *et al.*, 1973), amphibian (Wolf *et al.*, 1976; Lindsay and Hedrick, 1989) and a mammal (Moller and Wassarman, 1989).

Our experimental results in previous (Iwamatsu *et al.*, 1995) and present studies suggest that a chorion hardening enzyme(s) is released from the cortex into the perivitelline space during egg activation and exerts its action on the chorion from the inside. The isolation and purification of this enzyme(s) will be performed in further studies.

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