Electrophoretic Study on the Water Soluble Proteins of Dog Salmon Eggs

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(With 2 Text-figures)

In the eggs of many animals, the first visible change at fertilization is the breakdown of the cortical granules or alveoli found in the cortical ooplasmic layer of the unfertilized eggs. According to T. Yamamoto (1944a, b), some sort of invisible change propagates through the cortical ooplasmic layer prior to the breakdown of these cortical structures. The nature of this invisible change is however still obscure and the molecular basis of the breakdown of cortical structures is unknown. Monroy (1950) performed an electrophoretic study of the proteins of sea urchin eggs and found that the egg proteins undergo some sort of rearrangement immediately after fertilization. A similar observation was also reported by Ogawa and Kobayashi (1963) in newt eggs. These facts suggest that the rearrangement of egg proteins might be involved in the invisible cortical change. In fish eggs, however, Hamano (1957) failed to find a similar sign of the rearrangement of egg proteins after fertilization, though he detected a transient appearance of new protein after the parthenogenetic activation of the eggs. It seemed desirable to perform a study of the proteins of unfertilized, fertilized and parthenogenetically activated eggs by recent biochemical techniques. This is the first purpose of the present study.

Kanoh (1952) found that inseminated dog salmon eggs begin their embryonic development without the breakdown of cortical alveoli after immersion in isotonic $CaCl_2$ solution. He suggested that the alveolar breakdown itself is prevented in these eggs although the invisible change had propagated over the entire egg surface. It might be possible to consider that such inhibition of the alveolar breakdown is due to some change of the alveolar contents which might occur after immersion of the eggs in $CaCl_2$ solution. In order to decide whether this is correct or not, the chemical properties of the alveolar contents of unfertilized and of Ca-treated eggs were also examined in the present study.

Materials and Methods

Animals: Materials used in the present study were derived from the dog salmon, *Oncorhynchus keta*, captured at the Chitose Salmon Hatchery. Ripe

unfertilized eggs were obtained by dissecting the belly of mature females. In order to remove the coelomic fluid, these eggs were repeatedly washed immediately before use with Ringer's solution[1]. Insemination was performed by the dry method and the eggs were thoroughly washed with Ringer's solution to insure the penetration of spermatozoa into the eggs (T.S. Yamamoto, 1976) and to reduce the number of excess spermatozoa adhering to the egg surface. Although dog salmon eggs do not show any morphological change at the time of insemination in Ringer's solution, the egg activation can be induced independently of the entry of spermatozoa and occurs after the eggs are immersed in tap water (K. Yamamoto, 1951). The inseminated eggs reached 2 cell stage after 11 hours in tap water (10°C). In the present study, those eggs which showed over 90% cleavage after fertilization were used for the experiments. It is known that the inseminated eggs begin to develop without the breakdown of cortical alveoli after immersion in isotonic $CaCl_2$ solution instead of tap water (Kanoh, 1952; Kusa, 1953). For these experiments, unfertilized and fertilized eggs immersed in tap water or isotonic $CaCl_2$ solution for 0, 1/30, 1/4, 3 and 12 hours were used.

Preparation of protein solution: To avoid the denaturation of the egg proteins, the preparation was carried out at 1°C. Fifty eggs were rinsed with Ringer's solution and broken by cutting one by one with fine scissors. The cutting of all eggs of the same developmental stage was finished within 30 seconds. The sticky fluid obtained by cutting the eggs was diluted to a total volume of about 20 ml with Ringer's solution and was centrifuged at 1000 rpm for 10 minutes in order to separate free lipids and pieces of egg membrane from the fluid. After the removal of these buoyant constituents by aspiration and clean forceps, the yellowish orange fluid probably contained all substances other than oil drops from the egg cell. This fluid was then transferred into a cellulose tube (Visking Co., Germany), to which 20 ml of deionized water were added. After dialysis against 3 changes of deionized water for about 24 hours, the dialyzate was centrifuged at 10000 rpm for one hour. The clean supernatant fluid was filtered with paper and was lyophilized. By the procedure mentioned above, 40–60 mg of yellowish powder were obtained from the eggs treated with tap water. In the case of Ca-treated eggs, the yield was always less than that from tap water-treated eggs and was 20–35 mg. The protein solution for use was made by dissolving this powder in the buffer solution (pH 9.05) elaborated by Aronsson and Grönwall (1957, 1958) at a concentration of about 7 g/dl.

Electrophoresis: The protein solution was examined on "Separax" cellulose-acetate strips (20×110 mm, Jookoo Sangyo Co.). The sample was given on a line 30 mm from the cathodal end of the strips. Aronsson and Grönwall's buffer solution (pH 9.05) was used for the electrophoresis. The current was 0.4 mA/cm and the experiments were run for 3 hours at 4°C. After the run, the strips were stained in Ponceau 3R for the detection of proteins. In order to stain polysac-

1) Composition of Ringer's solution: M/7 NaCl 100 parts +M/7 KCl 2.8 parts +M/10 $CaCl_2$ 3.4 parts (pH 7.2).

charides, the strips were treated with the periodic acid-Schiff (PAS) method (Ogawa, 1971). After staining, the strips were dried for 24 hours at room temperature and cleared in liquid paraffin. The optical density was measured with a densitometer at 520 nm.

Results

The electrophoretic patterns of the water soluble proteins of unfertilized and fertilized eggs immersed in tap water for 0 (not activated), 1/30 and 12 hours are shown in Figure 1. All the components migrated anodically at the pH used. Seven main bands were recognized. It was found that the fast-moving bands *a*, *b* and *c* constitute about 60% of the total protein of the extract. No recognizable change in the number of bands or in the relative concentration of bands occurred after egg activation. The pattern obtained by the PAS stain is shown in Figure 1 E. Judging from the mobility, the fastest-moving band *a* and the slow-moving band *f* of the Ponceau-stained strips exhibited a positive PAS reaction.

If the egg activation without breakdown of cortical alveoli was induced in isotonic $CaCl_2$ solution, the electrophoretic pattern of the egg extract was to some extent altered (Fig. 2). In this case, too, all the components migrated anodically. Five bands were detected. The electrophoretic mobility of most of the components was apparently lower than that of tap water-treated eggs. In the eggs immersed for 1/30 hour, the fastest-moving component, band *h*, and the slowest-moving component, band *l*, were found in high concentrations. During further immersion in $CaCl_2$ solution, the relative concentration of band *h* in the extract gradually decreased but that of band *l* increased. Owing to the latter fact, bands *j* and *k* became very indistinct after 12 hours.

A positive PAS reaction was obtained in the bands *i*, *j* and *l*. Although the band *l* was conspicuous after 12 hours in the Ca-treated eggs, its relative concentration in the extract did not increase in the PAS-stained strips.

Discussion

The electrophoretic separation of the egg proteins of fishes has been performed by several investigators. Hamano (1957) carried out such experiment using Tiselius apparatus and found 7 components in the water soluble proteins from pond smelt eggs and 5 components in those from salmonid eggs. A paper electrophoresis of the water soluble proteins of fish eggs was carried out by Ohi (1962). He succeeded in separating *Oryzias* egg proteins into 5 components but obtained only 2 fractions from *Plecoglossus* and *Carassius* eggs. Ito *et al.* (1963) performed a zone electrophoretic study on starch grain block and revealed the existence of 3 components in the water soluble proteins of trout eggs. In a study on vitellogenesis of the zebrafish oocyte, Heesen and Engels (1973) carried out electrophoresis of the whole egg homogenate on cellulose acetate strips and

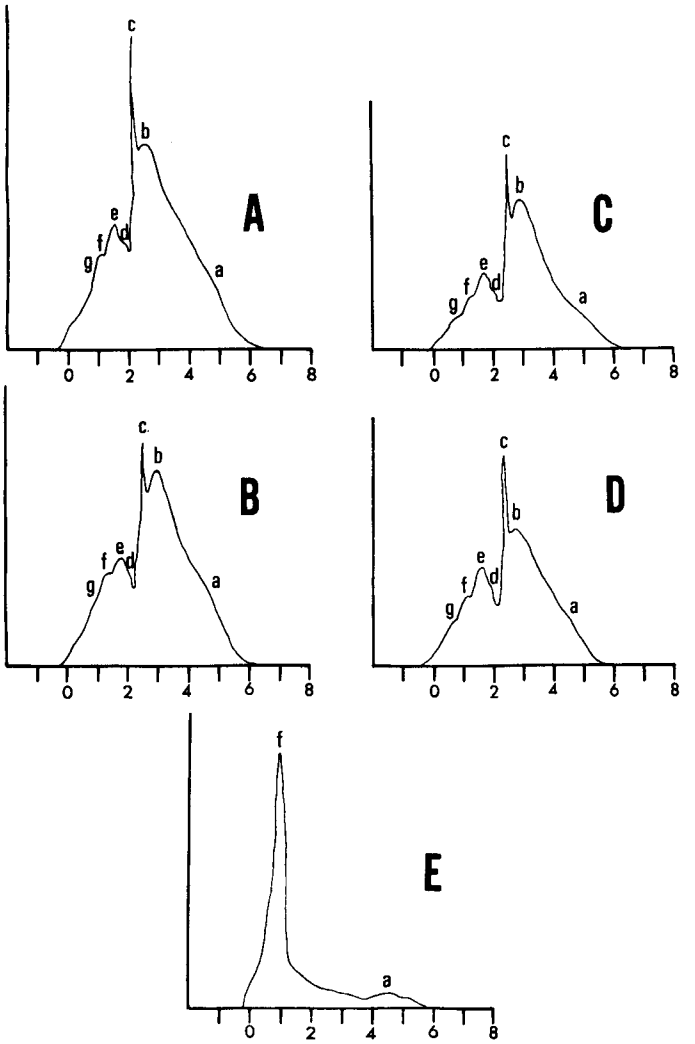

Fig. 1. Electrophoretic diagrams of extracts from unfertilized eggs immersed in tap water for 0 (not activated, A and E) and 1/30 (B) hours and from fertilized eggs immersed in tap water for 1/30 (C) and 12 (D) hours. Abscissa; distance (cm) from the point of application of samples (0). ordinate; relative density of staining. A-D, Ponceau 3R-stained pattern. E, PAS-stained pattern.

successfully separated its proteins into 7 components. In the present study, 7 components were separated from the water soluble proteins of dog salmon eggs. These results of separation were the same or better than those obtained by earlier workers.

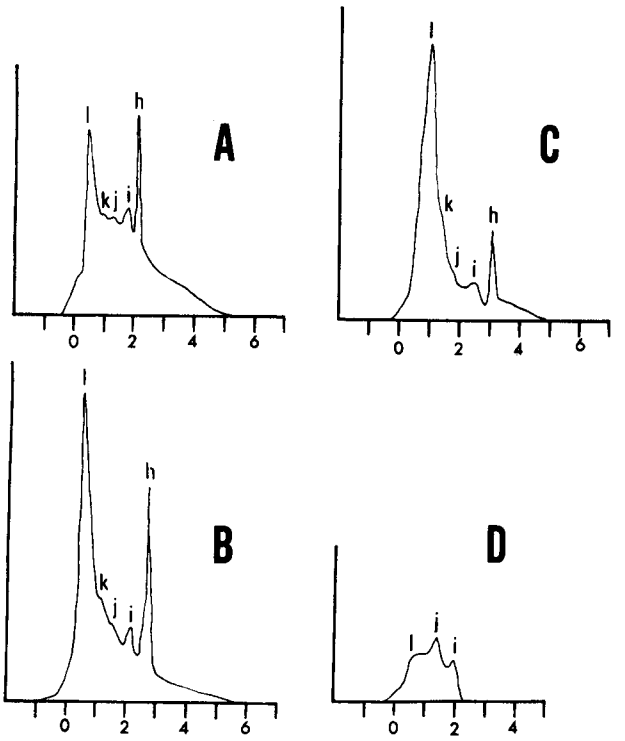

Fig. 2. Electrophoretic diagrams of extracts from fertilized eggs immersed in isotonic $CaCl_2$ solution for 1/30 (A), 1/4 (B and D) and 12 (C) hours. Abscissa and ordinate are the same as in Fig, 1. A-C, Ponceau 3R-stained pattern. D, PAS-stained pattern.

Hamano (1957) found a difference in the electrophoretic pattern between fertilized and parthenogenetically activated eggs of the salmonid and pond smelt. According to him, a peculiar component transitorily appears in parthenogenetically activated eggs but is never observed in fertilized eggs. This component seems to be formed by denaturation of the egg proteins which may occur *in vivo* immediately after parthenogenetic activation. In the present study, however, no difference in the electrophoretic patterns was observed between fertilized and parthenogenetically activated eggs. Such diverse results might be due to the variety of the method employed. Furthermore, differing from the cases of the sea urchin (Monroy, 1950) and newt (Ogawa and Kobayashi, 1963), no sign of rearrangement of the proteins was detected at the time of the initiation of embryonic development.

An intense PAS reaction was observed in the position of band *f*. Furthermore a weak reaction was obtained at the site of the indistinct band *a*. These facts indicate that these bands contain carbohydrate-protein complexes. In eggs

activated in isotonic $CaCl_2$ solution, the positive PAS reaction was obtained in the position of bands *i*, *j* and *l*. When band *f* of the tap water-treated eggs was compared with band *l* of the Ca-treated eggs, they were both slow-moving and PAS positive. These facts suggest that band *l* of the Ca-treated eggs was identical in its constituent with band *f* of the tap water-treated eggs. Heesen and Engels (1973) showed in zebrafish oocytes that two PAS positive bands appear after electrophoretic separation of the whole egg homogenate, their mobilities being different from each other. They identified the composition of the slow-moving and PAS positive band as the contents of yolk vesicles ("intravesicular yolk"). It has long been known that yolk vesicles appearing in the growing oocytes of fishes are the precursors of the cortical alveoli found in the cortical ooplasmic layer of ripe unfertilized eggs (Aketa, 1954; T.S. Yamamoto, 1955; Yamamoto and Oota, 1967). In the histochemical study of dog salmon eggs, Kusa (1951) reported that cortical alveoli show positive reactions to polysaccharides and protein tests. Considering these facts, it may be surmised that bands *f* or *l* of the present study represent the contents of cortical alveoli.

The electrophoretic mobility of most components of the egg proteins except the contents of the cortical alveoli (band *f*) decreased to some extent when eggs had been previously immersed in isotonic $CaCl_2$ solution. This fact indicates that the water soluble proteins of dog salmon eggs are readily united with Ca ions. Furthermore the relative concentration of band *l* in the extract increased as the duration of immersion in isotonic $CaCl_2$ solution was prolonged. In this connection it should be remembered that the lyophilized powder, obtained from the 50 eggs previously immersed in $CaCl_2$ solution, was apparently less in amount than that from tap water-treated eggs. Probably this fact indicates that most components of the egg proteins were united with Ca ions and could hardly dissolve in water. If fact, Yamaguchi (1958) reported that the egg proteins of dog salmon coagulate after the microinjection of isotonic $CaCl_2$ solution into the living egg. The contents of cortical alveoli seem to be not united with Ca ions, thus no change was induced in the electrophoretic mobility and solubility in water after immersion in $CaCl_2$ solution. These facts strongly suggest that bands *f* or *l* represent the contents of cortical alveoli. If so, the enhancement of the relative concentration of band *l* in the extract does not mean a real increase of this component in the eggs but is due to its unaltered solubility in water. Since the contents of cortical alveoli did not show any change in electrophoretic property in Ca-treated eggs, it may be supposed further that the inhibition of the breakdown of cortical alveoli in $CaCl_2$ solution is not due to changes in the physico-chemical property of the alveolar contents.

Heesen and Engels (1973) reported in zebrafish oocytes that the fast-moving and PAS positive band in their electrophoretic diagram represents egg yolk ("exogenous yolk"). In the present study, the fastest band *a* also showed a weak PAS reaction. This fact suggests that the component observed in the position of band *a* represents egg yolk. By the treatment of eggs with $CaCl_2$, the mobility of band

a was to some extent decreased indicating that yolk proteins possess an affinity to Ca ions. This fact coincides with Yamaguchi's observation that isotonic $CaCl_2$ solution injected into living salmon eggs causes the coagulation of egg proteins (Yamaguchi, 1958).

Summary

1. The water soluble proteins of the eggs of the dog salmon, *Oncorhynchus keta*, were separated by electrophoresis on cellulose acetate strips and seven bands were detected.

2. Up to 12 hours after fertilization or parthenogenetic activation of the eggs, no change in the electrophoretic pattern was detected.

3. The slow-moving band consisted of carbohydrate-protein complexes, the mobility of which did not alter after treatment of the eggs with isotonic $CaCl_2$ solution. It was suggested that this band represents the contents of cortical alveoli.

4. The fastest-moving band was also composed of carbohydrate-protein complexes. However its mobility considerably decreased after treatment of the eggs with $CaCl_2$ solution. It was surmised that this band represents yolk protein.

5. The mechanism underlying the inhibition of the breakdown of cortical alveoli in the eggs immersed in isotonic $CaCl_2$ solution was discussed in relation to the electrophoretic property of the alveolar contents.

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