DIRECT ISOLATION OF THE HYALINE LAYER PROTEIN RELEASED FROM THE CORTICAL GRANULES OF THE SEA URCHIN EGG AT FERTILIZATION

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ABSTRACT

Treatment of the eggs of the sea urchin with a 1 M solution of glycerol at fertilization allows the recovery from this solution of the protein released from the cortical granules, including that which would normally give rise to the hyaline layer. The calcium-gelable protein previously extracted from whole eggs and from isolated cortical material was found to be present in the glycerol solution, confirming its localization in the cortical granules and its role in the hyaline layer. Quantitative measurements on the eggs of two Hawaiian species, *Colobocentrotus atratus* and *Pseudoboletia indiana*, which have the widest variation in the gel protein content, demonstrated that a proportionate amount of this material was released at fertilization in these species, which correlates with the thickness of the hyaline layer in the two cases. In addition, the calcium-insoluble fraction of Sakai can be extracted from these eggs after removal of the hyaline protein by glycerol, showing that this is a different material. A simple method for the separation of the hyaline protein from the calcium-insoluble fraction in solution is provided.

INTRODUCTION

Early investigations into a possible protein precursor to the mitotic apparatus (MA) in sea urchin eggs led to the isolation of an unusual gel protein (5). This protein, which made up about 10% of the total cell protein in the species investigated, was extracted from the cells by dilute salt solution and formed a reversible gel on the addition of calcium, magnesium, or other divalent ions. However, its properties did not resemble those of any component then obtainable from the isolated MA (9), nor did it react with an antiserum against the dissolved MA (24); it remained a protein of unique properties but unknown function until recently.

The experiments of Sakai (16) suggested a possible cortical role for this protein, as his method for the isolation of the cortex was based on lysis of sea urchin eggs in 0.1 M $MgCl_2$, a condition which was known (5) to cause the gelation of this protein. However, Sakai's interest centered on the material extracted from the distilled water-washed cortices by 0.6 M KCl, which he termed "contractile protein," as this component showed sulfhydryl fluctuations over the division cycle. The material removed from the cortices by distilled water, which presumably included the protein of Kane and Hersh, was not studied as it showed no such fluctuation (17). Subsequently, he prepared a calcium-insoluble fraction from whole eggs (18) and from the isolated mitotic apparatus (19) and reported a thiol-disulfide exchange reaction between this fraction and the contractile protein of the cortex. Although Sakai (18) identified his calcium-in-

soluble fraction with the protein isolated by Kane and Hersh, the properties of the latter protein would predict, instead, that it would be found, at least in part, in the water-soluble fraction of the isolated cortex.

Investigations in our laboratory on the eggs of several species of Hawaiian sea urchins confirmed this prediction (6). Cortex isolated from unfertilized eggs in 0.02 M $CaCl_2$, which gave similar results as but better yields than $MgCl_2$, was found to contain a large fraction of the gel protein of the egg in several of the species studied. The use of a number of species demonstrated also that results from one species cannot be uncritically extrapolated to another, as the total amount of gel protein per milliliter of cells in the species studied varied by a factor of five, and the fraction of this protein in the isolated cortical material varied from 80 to 17%.

The localization of this protein in the cortical region in some species suggested that it might be present in the cortical granules, a possibility that was strongly supported by the work of Yazaki (25). She prepared a fluorescent antibody to the gel protein prepared by the method of Kane and Hersh (5) and showed that it stained the periphery of the unfertilized egg and the hyaline layer of the fertilized egg and reacted with hyaline material removed from the eggs by isotonic salt solution. The localization of this protein in the cortical granules and its subsequent appearance in the hyaline layer would be in agreement with the electron microscopic evidence on the origin of this layer from these granules (1, 2), and is also supported by comparisons of the amount of gel protein and the thickness of the hyaline layer in the eggs of several Hawaiian species (6). If this is the case, then Sakai's calcium-insoluble protein must have a different origin. Yazaki (25) clarified this by showing that Sakai's preparative method (18), based on the addition of calcium to dialyzed distilled water extracts of unfertilized eggs, yields not only the gel protein of Kane and Hersh, but an additional fraction which did not redissolve on dialysis against water. It is this latter fraction which showed sulfhydryl fluctuations during cell division, while the gel protein was inactive.

The physical properties of the gel protein have recently been studied in detail and the name hyalin proposed for it to simplify the terminology (21). One of the contributing factors to the confusion regarding its identity is the very marked dependence of its sedimentation coefficient on concentration, which makes unextrapolated coefficients of little value for identifying the protein in solution. Thus it appears, but cannot be confirmed, that the material isolated by Vacquier (22) is this protein.

The experiments described here had the following purposes: (*a*) to develop a method for the complete removal of the fertilization membrane and hyaline layer from fertilized eggs which would also permit recovery of unmodified hyalin and allow further cleavage of the blastomeres; (*b*) to quantitate the amount of hyalin obtained by this method from the two Hawaiian species, *Colobocentrotus atratus* and *Pseudoboletia indiana*, that differ most widely in their content of this protein. These measurements, when compared to the total hyalin content of these cells, will provide a direct demonstration of the fraction of this protein that is released from the cortical granules at fertilization; (*c*) to determine the effect of such treatment on subsequent cortex isolation. If the gel protein of the isolated cortex originates from the breakdown of the cortical granules, then cortices from such treated eggs should contain little or none of this material; (*d*) to determine the relation of hyalin to the calcium insoluble-protein(s) of Sakai (18). If, as the evidence appears to indicate, they are different components, then it should be possible to demonstrate this directly by obtaining the Sakai material from the eggs after the removal of hyalin by glycerol treatment; (*e*) to develop a simple method for separating the hyalin from other calcium-insoluble materials in solution.

MATERIALS AND METHODS

The eggs of the Hawaiian sea urchins *Colobocentrotus atratus* and *Pseudoboletia indiana* were used in these experiments. The gametes were obtained by the injection of isotonic KCl into the body cavity, the eggs were washed several times in sea water, and the jelly was removed by titrating the egg suspension to pH 4.8–5.0 with dilute HCl. The jellyless eggs were then washed and grown in Millipore filtered sea water (Millipore Filter Corp., Bedford, Mass.) containing 10^{-5} EDTA, pH 8.5, at 24°C. Egg extracts were made by homogenizing the eggs in 10 times their volume of solution with a Teflon glass homogenizer and centrifuging at 25,000 *g* for 20 min at 4°C. Dialysis was carried out for 24–48 hr at 4°C with continuous stirring. Extracts were made in distilled water or in 0.05 M NaCl, 0.01 M Tris, pH 7.5, referred to as NaCl—Tris solution in the text. Protein determinations were made by the

method of Lowry et al. (8), with a serum albumin standard. Calcium gels and precipitates were taken up directly in 0.5 M NaOH for protein determination. All protein measurements are given in terms of milligrams of protein per milliliter of packed, jellyless cells. Sedimentation studies were done in a Beckman Spinco Model E analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) equipped with Schlieren optics, phase plate and rotor temperature control. All ultracentrifuge runs were made at 20 ± 1°C.

RESULTS

Although the hyaline material of the blastomeres can be solubilized by a number of methods, its removal can be accomplished only in embryos lacking the fertilization membrane. Loss of this membrane does not occur naturally until the blastula stage, and in earlier embryos artificial means of removal must be used. Pretreatment of the eggs with proteolytic enzyme will prevent its formation on fertilization (15), and treatment immediately after fertilization will also remove it (23). Both of these methods, however, lead to the partial or complete loss of the hyaline layer by proteolytic action and thus are unsuitable for the investigations planned here. Treatment of the eggs with a molar solution of urea or other nonelectrolyte before fertilization is effective in preventing the formation of both the fertilization membrane and hyaline layer on insemination (10), and this was shown to be due to the stimulation of cortical granule breakdown in urea, followed by the dissolution of the fertilization membrane and the hyaline material (12, 13). To avoid possible complications from the parthenogenetic effects of urea, the author has used urea treatment immediately after fertilization as a reliable method of producing membraneless eggs (4). These eggs are generally without the hyaline layer also, as this material is solubilized by urea (14). Although this method of removal appears milder than treatment with proteolytic enzyme, experiments with purified hyalin showed that in the presence of 1 M urea its recovery from the solution by calcium gelation was reduced by about one-half. Since the effect on the fertilization membrane is nonspecific and depends only on the elimination of the ions of sea water while maintaining the osmotic balance (10), other nonelectrolytes can be substituted for urea. Glycerol, which was as effective as urea in the original experiments of Moore (10), proved equally effective with the Hawaiian species and did not influence the gelation of purified hyalin. To maintain the pH of the glycerol solution, which is of importance in its action (11), it is buffered at pH 8.3–8.5 with 0.005 M Tris.

Sperm is added to a measured volume (usually 1 ml) of jellyless *C. atratus* eggs in a large volume of sea water contained in a 50 ml centrifuge tube. The tube is agitated briefly, hand centrifuged to concentrate the eggs, and the sea water is replaced with a measured volume (usually 30 ml) of buffered 1 M glycerol solution. It is important that the eggs be suspended in glycerol solution at 30–40 sec after insemination, as fertilization membranes are elevated quite rapidly in this species. The eggs are allowed to remain in glycerol until 5 min after insemination, when they are gently hand centrifuged and the glycerol is replaced with sea water. If this step is done with care, the eggs do not clump and can be grown in normal rather than calcium-free sea water. At first cleavage the blastomeres separate into two spheres (Fig. 1), without evidence of hyaline or other restraining layer, while untreated blastomeres are very tightly apposed (6). *P. indiana* eggs are treated similarly, except that membrane elevation is slower in this species and the eggs are placed in glycerol at 60–75 sec after insemination. These eggs have little hyaline in the normal condition (6) and are without evidence of fertilization membrane or hyaline after glycerol treatment (Fig. 2). The cortical granules of unfertilized eggs of *C. atratus* break down in glycerol solution in the manner described by Moser (13), but those of the eggs of *P. indiana* do not, and hence these eggs must be fertilized to stimulate cortical granule breakdown.

After removing the eggs by hand centrifugation, the glycerol solution is chilled to 0°C and centrifuged at 25,000 *g* for 20 min to remove any material from broken eggs. In successful experiments this pellet is very small or absent; its size is a measure of egg breakdown. 2 M $CaCl_2$ is added to the centrifuged supernatant to give a final concentration of 0.02 M, the solution is allowed to stand for 15 min at 0°C, and the gel is collected by centrifugation at 1400 *g* for 2 min. After several washings with 0.01 M Tris containing 0.02 M $CaCl_2$ to remove any trapped soluble protein, the gel is either redissolved by dialysis against NaCl—Tris solution or taken up in 0.5 M NaOH for protein determination.

The glycerol solution used to treat the eggs of *C. atratus* at fertilization yields an average value of

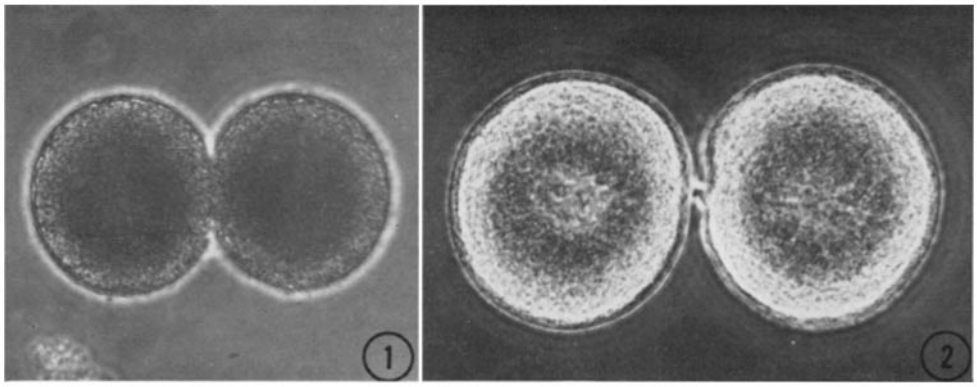

FIGURE 1 First cleavage of *C. atratus* eggs treated with glycerol at fertilization. Phase contrast. × 350.

FIGURE 2 First cleavage of *P. indiana* eggs treated with glycerol at fertilization. Phase contrast. × 350.

12 mg of hyalin per ml of eggs, which is somewhat higher than the average amount of this protein, 10 mg per ml of cells, that was recovered from the isolated cortex in this species (6). Extracts of glycerol-treated *C. atratus* eggs in NaCl—Tris solution contain an additional 3–4 mg of hyalin which can be gelled by calcium, to yield a total of 15–16 mg per ml of cells, in agreement with the total hyalin content of 15 mg per ml of eggs obtained by direct extraction with NaCl—Tris in previous experiments (6). Treatment of unfertilized eggs of *C. atratus* with glycerol solution causes cortical granule breakdown and also yields 12 mg of gel protein per ml of eggs. The glycerol solution used to treat *P. indiana* eggs after fertilization contains an average of 1.8 mg of gel per ml of cells. This is about one-half of the total hyalin content of 3.5 mg per ml of cells (6), and additional gel protein can be obtained by extraction of the treated cells in NaCl—Tris solution and gelation with calcium. As noted previously (6), total yields of hyalin in this species are more variable than in *C. atratus*, but with aliquots of one batch of eggs, the sum of the hyalin obtained from the glycerol and from the treated cells by extraction is close to the total yield obtained by direct extraction of untreated cells.

If the hyalin obtained from the isolated cortical material originates from the cortical granules, as previous experiments indicated, then the removal of this material by glycerol treatment should cause its disappearance from the isolated cortex. This was investigated with the eggs of *C. atratus*, chosen because of their much higher hyalin content. A control cortex isolation, by the method used previously (6), yielded a value of 12.4 mg of hyalin from the cortical material from 1 ml of eggs. Fertilization and glycerol treatment of a sample of eggs removed 12 mg of hyalin per ml of cells, and the cortical material isolated subsequently was much reduced in volume and contained only 0.4 mg of hyalin per ml of cells. Glycerol treatment thus removes the hyalin that would normally appear in the isolated cortex, demonstrating that this protein originates from the cortical granules.

If the calcium-insoluble material prepared by Sakai from distilled water extracts of eggs (18) contains components other than hyalin, as now seems clear (9), then this material should be precipitable from water extracts of fertilized eggs after removal of most of the hyalin by glycerol treatment. To test this, *C. atratus* eggs which had been fertilized and treated with glycerol were extracted in distilled water and the extract was dialyzed. These preparations often precipitate during dialysis but do form a dense white precipitate quite unlike the gelation of hyalin, on the addition of 0.02 M $CaCl_2$. The material precipitated spontaneously during dialysis appears to be part of the calcium-precipitable material, as the sum of protein precipitated during dialysis and on calcium addition is constant. The calcium precipitate is not completely dissolved by dialysis, confirming the observation of Yazaki (25), but if solubilized in 0.5 M NaOH, protein measurement gives a yield of 15 mg per ml of cells. Since only 3–4 mg of this protein is hyalin, more than 10 mg of this precipitate is nonhyalin material. The

addition of calcium to a dialyzed water extract of *P. indiana* eggs yields an average of 22 mg of precipitate per ml of eggs. Calcium-insoluble material from unfertilized and fertilized eggs is similar in amount, except for the presence of approximately 2 mg of additional hyalin in unfertilized extracts. Unlike the situation with *C. atratus*, however, much of the calcium precipitate in *P. indiana* can be dissolved by dialysis against NaCl—Tris solution, allowing ultracentrifugal examination. A total water extract of these eggs is a complex mixture in which the hyalin is visible (Fig. 3, lower trace) as a hypersharp peak, even though it makes up a relatively small fraction of the total extract. As in Sakai's preparations (20), the calcium-insoluble material precipitated from such an extract in the absence of salt or buffer does not represent a selective precipitation of any major protein component but contains many of the components of the whole extract, including the hyalin (Fig. 4, lower trace).

The hyalin of sea urchin eggs is usually prepared by extracting the eggs in NaCl—Tris solution and gelling the protein from the soluble fraction of the extract by the addition of calcium. This procedure yields relatively pure hyalin, as demonstrated in the upper trace of Fig. 4 and confirmed by dialysis and regelation. It can be shown that the action of the NaCl—Tris is that of causing selective precipitation of hyalin on the addition of calcium. If a water extract is made 0.05 M in NaCl and 0.01 M in Tris, the yield of precipitate on the addition of calcium is reduced from more than 20 to approximately 3 mg per ml of cells, and the insoluble material, after redissolving by dialysis, consists almost entirely of hyalin (Fig. 5, lower trace). This procedure removes the bulk of the hypersharp peak from the whole extract (Fig. 3, upper trace) and confirms the identification of this peak as hyalin. Further experiments show that the effect of the NaCl—Tris solution is due to the Tris buffer. Addition of 0.01 M Tris buffer alone to a

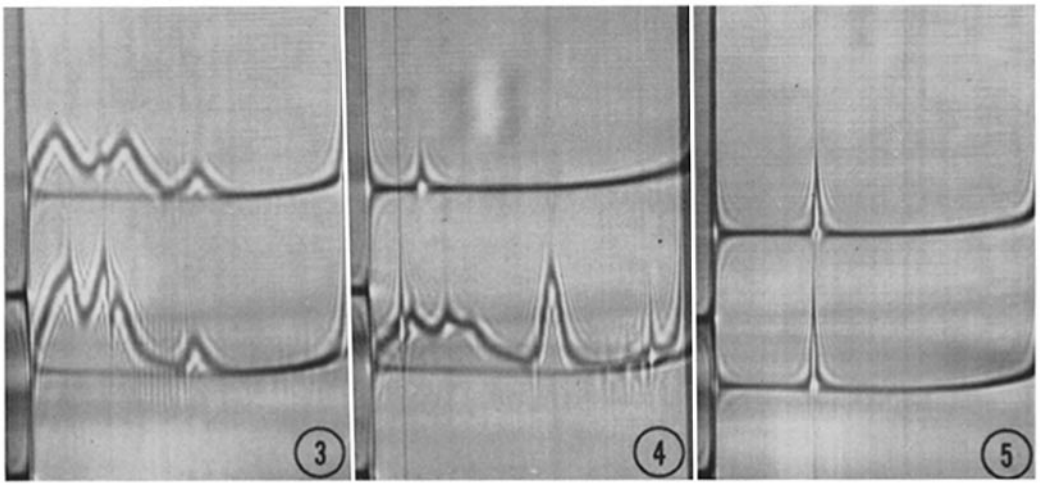

FIGURE 3 Lower trace: water extract of unfertilized *P. indiana* eggs, made 0.05 M in NaCl and 0.01 M in Tris. Upper trace: same extract after removal of 0.02 M calcium insoluble material. 20 min after reaching speed of 56,000 rpm, bar angle = 50°.

FIGURE 4 Lower trace: calcium-insoluble material from distilled water extract of unfertilized *P. indiana* eggs, redissolved by dialysis against 0.05 M NaCl, 0.01 M Tris. Upper trace: calcium-insoluble material from 0.05 M NaCl, 0.01 M Tris extract of unfertilized *P. indiana* eggs, redissolved by dialysis against same NaCl—Tris solution. 22 min after reaching speed of 56,000 rpm, bar angle = 50°.

FIGURE 5 Lower trace: calcium-insoluble material from distilled water extract of unfertilized *P. indiana* eggs made 0.05 M in NaCl and 0.01 M Tris before calcium addition, redissolved by dialysis against NaCl—Tris solution. Upper trace: calcium-insoluble material from same distilled water extract, made 0.01 M in Tris before calcium addition, redissolved by dialysis against NaCl—Tris solution. 52 min after reaching speed of 56,000 rpm, bar angle = 55°.

water extract before calcium addition is equally effective in causing the selective precipitation of hyalin (Fig. 5, upper trace), indicating that a slightly alkaline pH eliminates the precipitation of all other components on calcium addition.

DISCUSSION

These experiments provide independent evidence that the protein described by Kane and Hersh (5), now termed hyalin (21), is released from the cortical granules at fertilization to form part of the hyaline layer, confirming the immunological results of Yazaki (25). The two species used as experimental material, *C. atratus* and *P. indiana*, were chosen because their eggs had the greatest difference in the content of this protein, and in both cases a large fraction of the protein was recovered from the glycerol solution used to dissolve the products of cortical granule breakdown at fertilization. Previous experiments on cortex isolation in these species suggested that the gel protein found in the isolated cortical material originated from the cortical granules (6), and this has been confirmed in the present work. Removal of the hyalin by treatment with glycerol solution results in an almost complete absence of this protein from the isolated cortex, demonstrating that it has the same source.

The present experiments complete a comparison of the eggs of these species which extends from the purely chemical to the morphological. Extracts of unfertilized whole eggs showed the presence of several times as much gel protein in the eggs of *C. atratus* as in those of *P. indiana* (6), and the experiments reported here demonstrate that proportionate amounts of this protein are released from the cortical granules at fertilization. The much thicker hyaline layer present on the eggs of *C. atratus* as compared to *P. indiana* (6) provides visible evidence of the release of 12 mg of hyalin per ml of cells in the former species as compared to less than 2 mg per ml of cells in the latter. The recent experiments of Vacquier and Mazia (23) have emphasized the role of the hyaline layer in maintaining the apposition of the blastomeres of the sea urchin, and the much closer apposition of the blastomeres of *C. atratus* as compared to *P. indiana* at first cleavage (6) is thus probably a result of this difference in their hyaline layers. The very similar appearance of the blastomeres of these two species at first cleavage after removal of the hyaline layer lends support to such an interpretation.

The recovery of the protein hyalin from any procedure based on the breakdown of the cortical granules has not exceeded 80% of that extracted directly from unfertilized eggs in any of the species measured, and in some cases is considerably less. Although loss of smaller pieces of cortex could account for this in the case of "cortical" isolation methods, particularly with eggs such as *P. indiana* where this cortical material is very fragile, this explanation does not apply in the case of glycerol treatment. In these experiments the sum of the hyalin protein obtained from the glycerol and the treated eggs is equal to the total hyalin obtained by direct extraction, so the incomplete yield from the glycerol could be due either to less than 100% fertilization or less than 100% cortical granule breakdown in the fertilized eggs. The former explanation is unlikely as fertilization and cleavage approach 100%, so incomplete granule breakdown appears more likely, particularly since intact granules have been seen as late as blastula stage in some species (1), and occasional granules are seen in the eggs of the Hawaiian species at first cleavage. The difference in the fraction of total hyalin recovered from the glycerol in the two species studied might thus reflect a difference in the fraction of granules released at fertilization. The intact granules remaining after fertilization may be used to replace the hyaline layer, as its regeneration after removal by pronase (23) and by centrifugation (3) has been reported. However, as mentioned previously (21), the presence of some of this protein in the soluble phase of the cytoplasm cannot be discounted, but proof of this awaits a reliable method of preventing granule breakdown during extraction.

It now seems clearly established that the protein hyalin as defined (21) can be differentiated from the calcium-insoluble fraction of Sakai (18), prepared by the addition of calcium to dialyzed distilled water extracts of eggs. The latter material is a complex mixture of a number of components, of which the hyalin forms only a fraction. Previous preparations of hyalin obtained by the addition of calcium to NaCl—Tris extracts of eggs, on the contrary, consisted largely of a single component; the experiments reported here show that the addition of buffer before the addition of calcium provides a simple method of selectively gelling the hyalin from the more complex water extract. The preparation of a calcium-insoluble fraction from eggs after removal of the bulk of the hyalin by

treatment with glycerol solution demonstrates that this protein is not an obligatory component of this fraction; the absence of fluctuations in the state of its sulfhydryl groups during cell division (25) eliminates its having any role in the thiol-disulfide interchange reported by Sakai (18). The active component of the calcium-insoluble fraction of Sakai may be the microtubular protein, as this can be precipitated by calcium (7); whether this method can provide a useful technique for the isolation of this protein awaits further investigation.

In addition to providing a simple method for the quantitation of the hyalin released at fertilization, the glycerol procedure should be useful for other studies. The material from the cortical granules which contributes to the formation of the fertilization membrane (1, 2) must also be present in the glycerol solution and should form the bulk of the protein remaining in this solution after removal of the hyalin. Recovery and analysis of this material should be of considerable value in understanding the mechanism of fertilization membrane formation.

This investigation was supported by United States Public Health Service Grant No. GM-14363.

Received for publication 5 November 1969, and in revised form 19 December 1969.

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