RE-INITIATION OF MEIOSIS IN MYTILUS OOCYTES WITH ACROSOME REACTION PRODUCT OF SPERM

BULL. MAR. BIOL. STN. ASAMUSHI, TÔHOKU UNIV., 18(1), 11-23, 1985

RE-INITIATION OF MEIOSIS IN *MYTILUS* OOCYTES WITH ACROSOME REACTION PRODUCT OF SPERM[1)]

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In the marine mussel (*Mytilus edulis*) oocytes meiosis was re-initiated with sperm product obtained after acrosome reaction. The meiosis-inducing factor was proteinous substance with an apparent molecular weight of 18,000 or 22,000, containing saccharides. The activity was distinguished in heat stability from egg membrane lysin. The factor seems to inactivate an inhibiting factor of oocyte surface.

In many invertebrates, spermatozoa undergo acrosome reaction prior to penetration into eggs. During the acrosme reaction, the contents of the acrosomal vesicle are discharged into surrounding medium and the inner membrane of the acrosomal vesicle is extruded. Egg membrane lysin (COLWIN and COLWIN, 1958; HAINO and KIGAWA, 1966) and bindin (VACQUIER and MOY, 1977; BRANDRIFF, MOY and VACQUIER 1978) are known as physiological active substances derived from the acrosomal vesicle. A basic substance extracted from sea urchin or polychaete sperm parthenogenetically activates eggs (MOTOMURA, 1954; OSANAI, 1964, 1976). The acrosomal vesicle contains basic proteins (DAS, MICOU-EASTWOOD and ALFERT, 1975; BRANDRIFF, MOY and VACQUIER, 1978). These findings suggest a possibility that the acrosomal vesicle contains factor(s) relating to egg activation. This suggesion led us to search unknown physiological activity in sperm products after acrosome reaction.

The acrosome reaction of *Mytilus* sperm is artificially induced by the addition of excess calcium (WADA *et al.*, 1956). The supernatant of acrosome-reacted sperm is considered to contain substances derived from the acrosomal vesicle. We call this supernatant as "acrosome reaction product (ARP)". ARP is known to have egg membrane-lysing activity (DAN, 1962). The present investigation shows the presence of a new physiological activity, meiosis-inducing activity, in the ARP.

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Materials and Methods

Gametes

Marine mussels, *Mytilus edulis*, were collected in Mutsu Bay, Aomori. Sperm were obtained by dissecting testes. Extruded sperm were suspended in small amount of sea water. The sperm suspension was filtered through the gauze to remove tissue fragments and then centrifuged at 3,500 rpm for 10 minutes. The supernatant was aspirated off and the remained sediment was used as the stock of sperm.

Eggs (primary oocytes at the first metaphase of meiosis) were collected by electric stimulation according to Iwata (1949). *Mytilus* females were stimulated for 10–15 seconds with a simplified apparatus for the induction of sea urchin spawning (Osanai, 1975). The spawning began about 1 hour after stimulation. Discharged oocytes were collected with a pipette.

Sea water

Artificial sea water was prepared according to modified Herbst's procedure (Osanai, 1975). Ca, Mg-free sea water was prepared by substituting $CaCl_2$ to NaCl and $MgSO_4$ to Na_2SO_4. Ca, Mg-free EDTA sea water was Ca, Mg-free sea water containing 0.1 mM ethylenediamineteraacetic acid and adjusted to pH 8 by NaOH.

Preparation of acrosome reaction product

Stock sperm was diluted in ninefold volume of sea water. One part of M/3 $CaCl_2$ was added to 9 parts of the diluted sperm suspension. The scanning electron microscopy showed that the majority of spermatozoa underwent acrosome reaction (Fig. 1). The $CaCl_2$-added sperm suspension was centrifuged with 10,000 g for 20 minutes at 4°C. The supernatant was dialyzed against sea water overnight to remove excess $CaCl_2$. The remained dialyzate (crude acrosome reaction product; cARP) was preserved in a freezer at −20°C until use.

SDS-polyacrylamidegel electrophoresis

Crude acrosome reaction product (cARP) and fractions obtained in the process of purification of ARP were applied to electrophoresis. Soluble fractions were precipitated by adding equal volume of cold 20% tricholoroacetic acid. The pricipitated samples (1 mg/ml) were dissolved in 0.01 M phosphate buffer (pH 7.2) containing 1% sodium dodecylsulphate, 0.01% N,N,N′,N′-tetramethyl-ethylenediamine, 25% glycerol, 1% β-mercaptoethanol. Then these solutions were heated at 100°C for 3 minutes. Samples were applied to 15% SDS-polyacrylamidegel electrophoresis (at pH 7.2, supplied current 8 mA per tube). After the electrophoresis, the gels were stained in 0.05% cromassie blue for proteins.

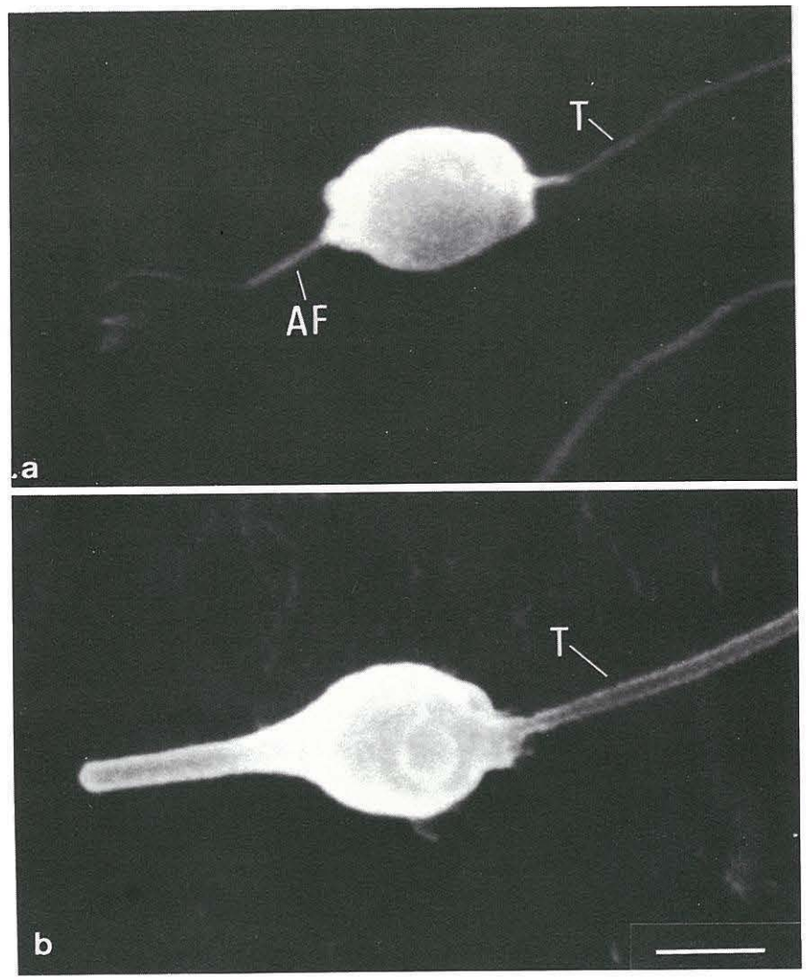

Fig. 1. Scanning electron micrographs of *Mytilus edulis* spermatozao before (b) and after acrosome reaction (a). AF: acrosomal filament: T: tail. The bar indicates 1 μm.

Bioassays

Oocytes (at the first metaphase of meiosis) were exposed to test media and then placed in ordinary sea water for 1 hour. Polar body formation was used as the criterion of re-initiated meiosis. The polar bodies extruded on egg circumference could be observed, but on upper and lower surface could not. Actual ratios of polar body formation must be higher than the values shown in the present papar.

To test egg membrane lysin activity, NaCl (58 mg/ml) was added to test media.

The egg membrane could be made visible by plasmolysis in the hypertonic media.

Experiments and Results

Meiotic maturation in normally fertilized oocytes

The oocytes spawned by electric stimulation were at the first metaphase of meiosis. They were ellipsoidal, about 65 μm in long axis and about 55 μm in short axis, and surrounded by a thin envelope, about 1 μm in thickness. Within 5 minutes after insemination, the fertilized eggs became spheroidal. They extruded the first and second polar bodies 30 minutes and 45 minutes after fertilization,

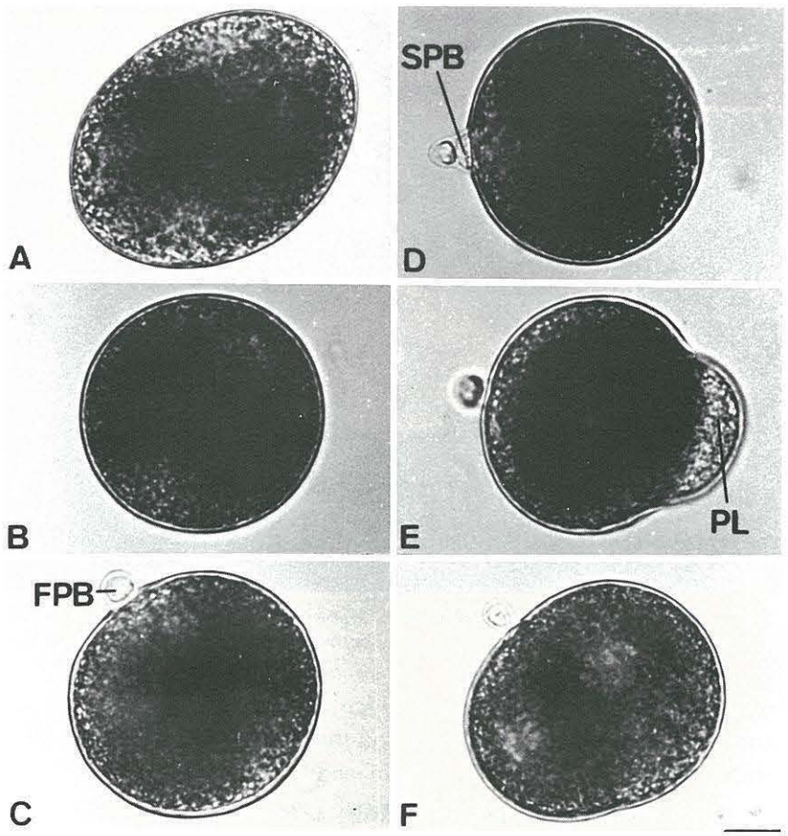

Fig. 2. Early development of *M. edulis* eggs following fertilization. A : unfertilized oocyte. B : spheroidal oocyte, 5 minutes after insemination. C : the first polar body (FPB) was formed, 30 minutes. D : the second polar body (SPB) was formed, 45 minutes. E : the polar lobe (PL) appeared at the vegetal pole, 90 minutes. F : Two-cell stage, 100 minutes after insemination. The bar indicates 10 μm.

respectively. The first cleavage occurred during 1.5 hours (Fig. 2).

Meiosis of unfertilized oocytes in cARP

Unfertilized oocytes were suspended in cARP. The oocytes transformed from ellipsoidal to spheroidal and extruded the first polar body. The time course of this change was similar to the fertilized oocytes. In cARP the oocytes often extruded

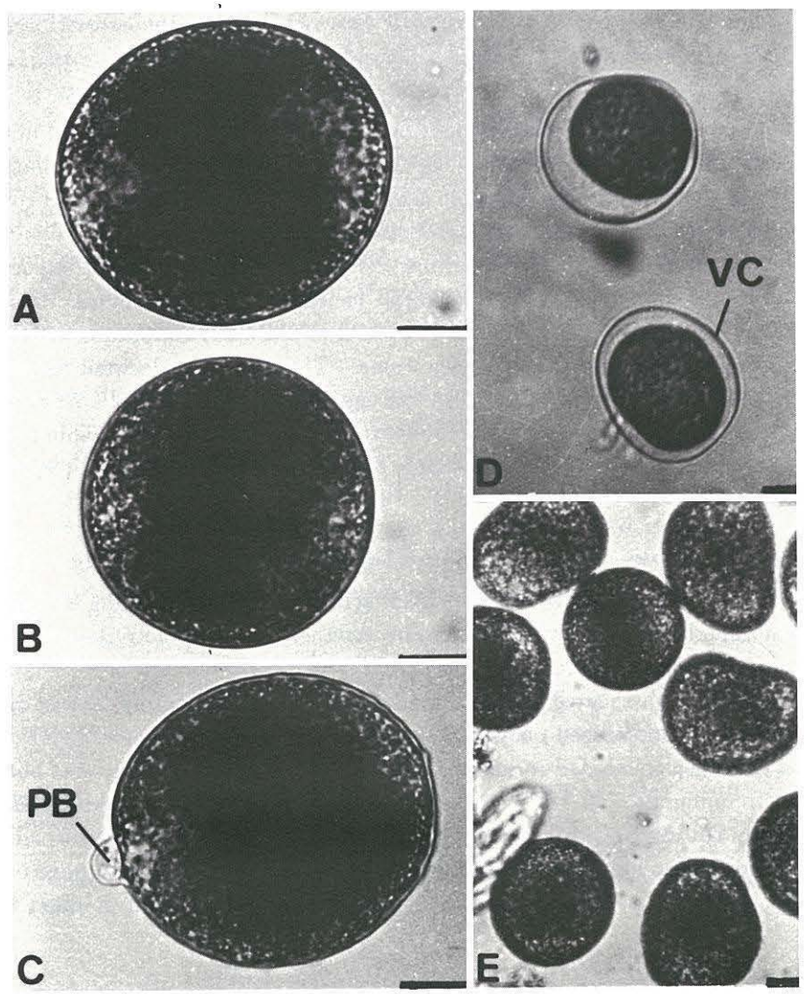

Fig. 3. Meiosis-induction and membrane lysis by cARP. Unfertilized oocytes (A) was exposed to cARP for 10 minutes and then incubated in sea water. Immediately, the oocytes became spheroidal (B). The polar body formed after 30 minutes (C). Oocytes were suspended in hypertonic cARP. The egg envelope rendered visible (D) after 10 minute inculation, but disappeared after 1 hour (E). The bars indicate 10 μm.

Table 1.
Meiosis-induction with cARP

Concentration (Dilution)	1*	1/2	1/4	1/8	1/16	1/32	0
Polar body formation (%)	38.7	35.6	20.7	5.3	0.2	0.2	0.0

* The stock solution of cARP (4.4 mg protein/ml) was diluted with sea water. Oocytes were exposed to sea water containing cARP for 10 minutes and then incubated in sea water for 1 hours.

the second polar body, but did not cleave. When the oocytes were placed in cARP for a long time, the egg envelope was dissolved (Fig. 3E) and the polar body separated away from the egg surface. This observation shows that cARP contains meiosis-inducing activity and egg membrane lysin activity.

The separation of polar bodies from the egg surface by a longer exposure to cARP caused a difficulty for the scoring of meiosis-reinitiated oocytes. To avoid the difficulty, unfertilized oocytes were exposed to sea water containing cARP for a limited duration and then placed in sea water. The oocytes underwent meiosis and the extruded polar bodies remained on the egg surface (Fig. 3B, C). After 1 hour incubation the percentages of polar body formation were scored (Table 1). The result shows that the meiotic maturation is parthenogenetically induced by the brief exposure.

Separation of meiosis-inducing activity and egg membrane lysin activity

The cARP contained two physiological activites, meiosis-inducing activity and egg membrane lysin activity. To examine whether these two activiteis were separable, their chemical natures were compared.

Crude ARP was heated at 100°C for 5 minutes. After cooling, it was divided into two lots. Unfertilized oocytes were exposed to the first heated cARP for 10 minutes and then incubated in sea water for 1 hour. The polar body was observed to the circumference of 18.3% eggs (Table 2). Hypertonic heated cARP was prepared by adding NaCl to the second lot. Unfertilized oocytes were suspended in the hypertonic heated cARP to test membrane lysin activity. After 1 hour incubation, the egg envelope separated from the egg surface, but remained intact. This

Table 2.
Meiosis-induction with heated cARP (100°C, 5 min.)

Concentration (Dilution)	1	1/2	1/4	1/8	1/16	1/32	0
Polar body formation (%)	18.3	12.0	10.6	2.1	3.3	0.5	0.0

resut indicates that the egg membrane lysin is heat labile and the meiosis-inducing factor is relatively heat stable.

Crude ARP was precipitated by dialysis against distilled water. The dialyzate containing a white precipitation was centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant was dialyzed again in sea water to restore salinity. The precipitate was dissolved in the ordinary volume of sea water (equal to the volume of the biginning cARP). Unfertilized oocytes were suspended in these solutions for bioassay. Miosis-inducing activity was stronger in the precipitate fraction than in the supernatant fraction (Table 3). Membrane lysin activity was detected only in the preciptate fraction.

Crude ARP was precipitated by the addition of fourfold volume of cold ethyl alcohol (−20°C). The precipitate was dissolved in the oridinary volume of sea water and then dialyzed against sea water. The dialyzate was used to bioassay. Oocytes underwent meiosis and the egg envelopes were dissolved in sea water containing the alcohol precipitate (Table 4). A white precipitate of cARP obtained by 40% saturation with ammonium sulfate was dissolved in the ordinary volume of sea water. This solution induced polar body formation and dissolved the egg envelope (Table 5).

These results show that the meiosis-inducing factor is proteinous and is distinguished in heat stability from the egg membrane lysin described by BERG (1950).

Meiosis-inducing activity was pH dependent. Unfertilized oocytes were exposed to cARP adjusted to various pHs by adding 0.1 N HCl or 0.1 N NaOH. After the exposure for 10 minutes, the oocytes were incubated in sea water. The maximum activity was observed at pH 8.6 (Fig. 4). The oocytes incubated in cARP-absent solutions of pHs 4-10 remained unchanged.

Table 3.
Meiosis-inducing activity of cARP after dialysis against distilled water

Concentration (Dilution)		1	1/2	1/4	1/8	1/16	1/32	0
Polar body formation (%)	sup.*	2.2	2.2	1.3	0.9	3.0	0.0	0.0
	ppt.**	32.1	27.3	9.5	0.2	0.3	0.0	

* The supernatant was dialyzed against sea water and** the precipitate was dissoved in sea water.

Table 4.
Meiosis-induction with the ethanol precipatate of cARP

Concentration (Dilution)	1	1/2	1/4	1/8	1/16	1/32	0
Polar body formation (%)	35.2	30.4	13.9	5.1	5.0	3.5	0.0

Table 5.
Meiotic induction with the precipitate by 40% saturation with ammonium sulfate

Concentration (Dilution)	1	1/2	1/4	1/8	1/16	1/32	0
Polar body formation (%)	32.2	17.0	20.2	22.3	0.9	0.0	0.0

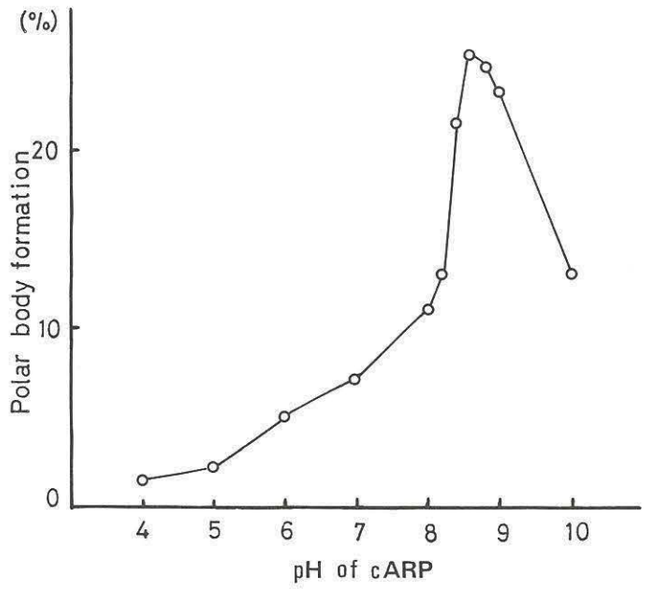

Fig. 4. Effect of pH on meiosis-inducing activity of cARP.

Purification of meiosis-inducing factor

Crude ARP (Fraction A) was salted out by 30% saturation with ammonium sulfate. The supernatant (Fraction B) formed a precipitate by the dialysis against distilled water. The precipite was dissolved in sea water and then mixed with the fourfold volumes of cold ethyl alcohol (−20°C). Newly formed precipitate (Fraction C) was dissolved again in sea water and filtered through a Sephadex G-50 column (Fraction D). The fractions, A, B, C and D, which had meiosis-inducing activity, were frozen and stored until use.

Crude ARP and the partially purified fractions were applied to SDS-polyacrylamidegel electrophoresis. The Sephadex G-50 column effluent (Fraction D) was separated to two bands with apparent molecular weights of 18,000 and 22,000 (Fig. 5). This fraction showed both meiosis-inducing and membrane lysin activities.

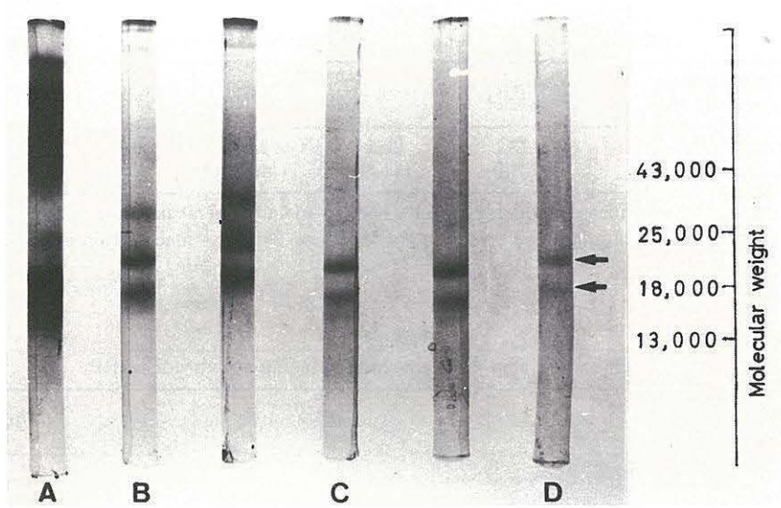

Fig. 5. Disc electrophoresis of ARP fractions, A, B, C and D (see text).

Table 6.
Meiosis-induction with aARP

Concentration (Dilution)	1*	1/2	1/4	1/8	1/16	1/32	0
Polar body formation (%)	72.5	70.3	68.3	61.1	25.3	16.6	0.0

* The stock solution (1.02 mg protein/ml) was diluted with sea water.

Rapid addition of ethyl alcohol to cARP at room temperature caused heat production, resulting in the inactivation of egg membrane lysin. The alcohol precipitate was saturated in sea water. The saturated solution contained 1.02 mg/ml proteins which was determined by the Biuret method (Lowry *et al.*, 1951). In this solution many oocytes (72.5%) formed the polar body, which remained on the egg surface without being separated away (Table 6).

Effect of protease and periodate on meiosis-inducing factor

The saturated solution of alcoholic cARP precipitate (1.02 mg proteins/ml) was mixed with 0.001-0.05% trypsin for 1 hour at room temperature and then heated at 100°C for 5 minutes to inactivate the protease. The meiosis-inducing activity decreased with the amount of added trypsin (Table 7).

Miosis-inducing factor was inactivated by the treatment with periodic acid. Periodic acid (0.1-100 μM) was added to the saturated alcoholic cARP solution and

Table 7.
Effect of trypsin on the meiosis-inducing activity of aARP

Concentration of trypsin (%)	0.0	0.001	0.005	0.01	0.05
Polar body formation (%)	73.1 (51.0)	20.1 (11.3)	3.6 (4.9)	0.0 (0.0)	0.0 (0.0)

The aARP was incubated with trypsin for 1 hour at room temperature prior to bioassay. The parenthesized numbers show polar body formation after the inactivation of trypsin by heating.

Table 8.
Effect of periodic acid on the meiosis-inducing activity of aARP

Concentration of periodic acid (M)	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Polar body formation (%)	69.5	10.8	5.3	0.0	0.0

after 30 minutes the mixture was dislyzed against sea water to remove periodate. Unfertilized oocytes were incubated in the dialyzate for 1 hour. Miosis-inducing activity decreased with the dose of periodic acid (Table 8).

Inhibition of meiosis-inducing activity with egg extract

Mytilus oocytes formed parthenogenetically the polar body in Ca, Mg-free sea water containing 10 mM EDTA and in isotonic solution of nonelectrolytes, such as urea, glycine and glucose. Since the oocytes were cytolyzed by prolonged incubation in these media, they were returned to sea water after a limited exposure and placed in sea water for 1 hour until the scoring of polar body formation. The polar body formation was induced also by the brief treatment (Table 9, Fig. 6).

Mazia *et al.* (1975) suggested that Ca, Mg-free sea water and nonelctrolyte solutions removed a factor inhibiting egg activation from the egg surface. If the media examined in the present experiments removed an inhibiting factor from *Mytilus* oocytes, the released factor would inhibit the meiosis-inducing activity of ARP.

Concentrated oocyte suspension (10 ml) was suspended in 90 ml of Ca, Mg-free EDTA sea water for 15 minutes and then the oocytes were settled. The supernatant was dialyzed against distilled water. The precipitate in the dialyzate was collected and stocked in ethyl alcohol (ES). The precipitate of cARP obtained by dialyzing against distilled water overnight was also preserved in ethyl alcohol. The alcoholic precipitate of cARP (4 mg wet weight/ml) was dissolved in sea water (aARP). Oocytes were suspended in the mixture of ES and aARP for 1 hour and polar body formation was scored. Miosis-inducing actvity decreased with the amount of egg

Table 9.
Meiosis-induction with Ca, Mg-free media

Treatment	Polar body formation (%)
Ca, Mg-free EDTA a.s.w. (15 min.)	45.0
1M Urea (2 min.)	20.1
1M Glycine (2 min.)	25.5
1M Glucose (15 min.)	27.0
aARP (1 hr.)	72.5
Control	0.0

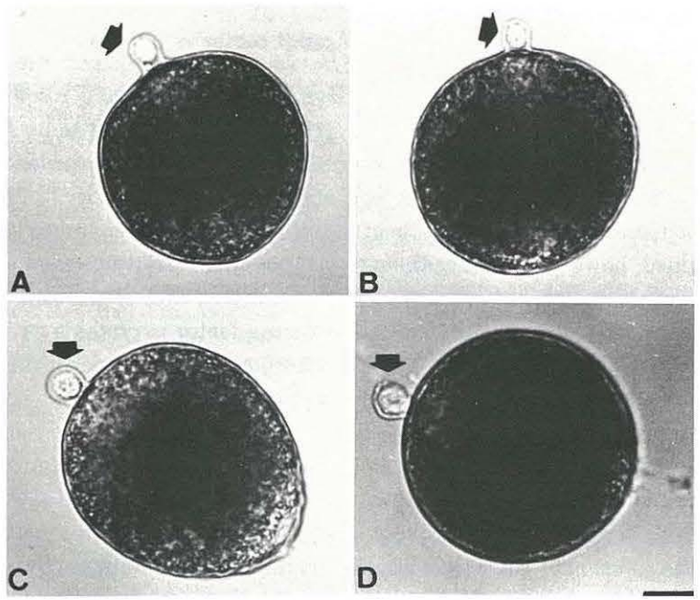

Fig. 6. Meiosis-induction in *M. edulis* oocytes with Ca, Mg- free media. Unfertilized oocytes were exposed to Ca, Mg-free EDTA sea water for 15 minutes (A), to 1 M urea for 2 minutes (B), to 1 M glycine for 2 minutes (C) or to 1 M glucose for 15 minutes (D) and then incubated in sea water for 1 hour. The arrows indicate polar bodies. The bar indicates 10 μm.

extract (ES) (Table 10). The oocytes did not extrude the polar body in sea water containing the ES alone. This result shows that an egg factor extracted from unfertilized *Mytilus* oocytes inhibits the meiosis-inducing activity of ARP.

Table 10.
Effect of egg extract (ES) on the meiosis-inducing activity of aARP

aARP (mg/ml)	ES (mg/ml)	Polar body formation (%)
0	0	0.0
4	0	58.7
4	0.1	50.6
4	0.5	18.2
4	1	4.0
0	1	0.0

Discussion

By being exposed to excess calcium, *Mytilus* sperm undergo acrosome reaction, releasing egg membrane lysin (Wada *et al.*, 1956; Dan, 1962). The present examination shows that the supernatant of acrosome-reacted sperm suspension contains a meiosis-inducing activity besides the egg membrane lysin activity. The meiosis-inducing factor is distinguished by heat stability from the membrane lysin. It is not ascertained, however, whether the meiosis-inducing activity owes to a substance different from the membrane lysin or to different active sites of the same substance. Electrophoresis indicates that the meiosis-inducing factor is proteins with apparent molecular weights of 18,000 and 22,000. The inactivation experiment with periodate suggests that the active substance contains saccharides.

Mytilus oocytes are spawned at the first metaphase of meiosis and remain in this stage until the activation by sperm or parthenogenetic agents. Re-initiation of meiosis is parthenogenetically induced not only with the miosis-inducing factor obtained from acrosome reacted sperm, but also with Ca, Mg-free sea water and nonelctrolyte solutions. Meiosis is arrested at the second metaphase by a cytostatic factor in amphibian oocytes (Masui *et al.*, 1980). It is not ascertained whether an inhibitor, such as the cytostatic factor, arrests meiotic maturation in *Mytilus* oocytes. However, the re-initiation of meiosis by Ca, Mg-free sea water and nonelectrolytes suggests the removal of the inhibitor from the oocytes. It seems to be a possible explanation that the meiosis-inducing factor obtained from *Mytilus* sperm inactivates the inhibitor located on the egg surface or in the egg cytoplasm, because the miosis-inducing activity is reduced by the egg extract.

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