[Chem. Pharm. Bull. 26(2) 405—410 (1978)]

UDC 557.156.04 : 615.35.011.4.076.9

The Change of Solubility Properties of Zona Pellucida to Proteases Related to Fertilizability of Mouse Ova *in Vitro*

Shigeru Aonuma, Masaru Okabe, Yuichi Kawai, and Makoto Kawaguchi

Faculty of Pharmaceutical Sciences, Osaka University[1]

(Received May 12, 1977)

Zona solubility properties to proteases were found to be different depending on the surroundings in which the treatments were done. The authors found that not all mouse ova which ovulated after the usual superovulation treatment were susceptible to zona removal by proteases in modified Krebs Ringer bicarbonate medium, that this susceptibility diminished as the time between ovulation and recovery increased, and that the proportion of fertilizable ova *in vitro* was the same order and the same pattern as the changes of zona solubility properties. The additional treatment of the mice with PMS abolished the decline in susceptibility and fertilizability. When ova were recovered at 12 hr after HCG injection and held in culture for a further 12 hr, there was no significant decline in either variable. If ova were placed for 2 hr in sperm suspension, dissolution of the zona by protease was completely prevented but this effect was not produced by sperm-free supernatants of sperm suspension. The effect was induced sooner by *in vitro* capacitated spermatozoa (15 min) than by epididymal spermatozoa (45 min). The loss of dissolution susceptibility in these treatments with sperm suspension was associated with the time of sperm penetration reported in previous paper[2].

Keywords——zona reaction; *in vitro* fertilization; zona lysis; aging of ova; sperm penetration

It was discovered independently by Austin[3] and Chang[4] that rat and rabbit spermatozoa must be exposed to the female reproductive tract for sometime before they acquire the ability to penetrate the ovum. The results of subsequent studies have suggested that a similar process may take place in hamster,[5] ferret,[6] cat[7] and mouse[8]. The process of sperm capacitation is associated with the attainment of the ability of sperm to penetrate the zona pellucida which surrounds the ova[9] and the occurrence of the zona reaction in response to penetration by the fertilizing sperm in many mammals represents block to subsequent capacitated sperm penetration.[10] It has been demonstrated that the substantial alterations occur in the solubility properties of the zona pellucida associated with the zona reaction.[11]

Krzanowska[12] reported that the time of zona removal with trypsin in the unfertilized ova collected one day after ovulation needed longer period than that of the day of ovulation.

1) Location: *133–1 Yamadakami, Suitashi, Osaka, Japan.*
2) S. Aonuma, M. Okabe, and Y. Kawai, *Yakugaku Zasshi*, **96**, 1307 (1976).
3) C.R. Austin, *Aust. J. Biol. Sci., Ser. B.*, **4**, 581 (1951).
4) M.C. Chang, *Nature, London*, **168**, 697 (1951).
5) M.C. Chang and D. Sheaffer, *J. Hered.*, **48**, 107 (1957).
6) M.C. Chang and R. Yanagimachi, *J. Exp. Zool.*, **154**, 175 (1963).
7) C.E. Hamner, L.L. Jennings, and N.J. Sojka, *J. Reprod. Fert.*, **23**, 477 (1970).
8) Y. Toyoda, M. Yokoyama, and T. Hoshi, *Jap. J. Anim. Reprod.*, **16**, 147 (1971).
9) A. Pavlok and A. Mclaren, *J. Reprod. Fert.*, **29**, 91 (1972).
10) A.W.H. Braden, C.R. Austin, and H.A. David, *Aust. J. Biol. Sci.*, **7**, 391 (1954).
11) *a*) M.C. Chang and D.M. Hunt, *Exp. Cell. Res.*, **11**, 497 (1956); *b*) M. Domon, M.C. Pinsker, and B. Mintz, *Biol. Reprod.*, **9**, 246 (1973); *c*) K. Gould, L.J.D. Zaneveld, P.N. Srivastava, and W.L. Williams, *Proc. Soc. Exp. Biol. Med.*, **136**, 6 (1971); *d*) R.B.L. Gwatkin, *J. Reprod. Fert.*, **7**, 99 (1964); *e*) M. Inoue and D.P. Wolf, *Biol. Reprod.*, **11**, 558 (1974); *f*) B. Mintz and J.D. Gearhart, *Dev. Biol.*, **31**, 178 (1973); *g*) M. Smithberg, *Anat. Rec.*, **117**, 554 (1953).
12) H. Krzanowska, *J. Reprod. Fert.*, **31**, 7 (1972).

This suggests that the ova may undergo zona reaction-like change on the zona pellucida without contribution of spermatozoa. On the other hand, Iwamatsu and Chang[13] reported that the fertilizing ability of mouse ova changed during maturation. To investigate the relationship between biological change and physicochemical properties of zona, the penetration of sperm capacitated *in vitro* into the zona pellucida was observed on the ova collected from one side of the superovulated female oviduct various time after superovulation, and simultaneously the enzymatic solubility properties of zona were studied in modified Krebs Ringer bicarbonate medium using the ova from the other side of the oviduct.

Experimental

Four to five-week-old virgin ddy mice were superovulated by the injections of 5 IU of PMS (Teikoku Zoki) and of 5 IU of HCG (Teikoku Zoki) 48 hr apart. Animals were sacrificed by cervical dislocation 12—24 hr after the administration of HCG. The ova surrounded with cumulus mass were released from excised oviducts into the culture medium by puncturing the ampullar region.

Zona Solubility Properties——Zona solubility properties were investigated in following medium; 1) Phosphate buffered medium, pH 7.2,[12] containing 4 mg/ml bovine serum albumin (Sigma; Fr. V) (m-KRP). 2) Modified Krebs Ringer bicarbonate medium containing 4 mg/ml bovine serum albumin[8] (the amount of $NaHCO_3$ was modified to 5.0 g/l) was equilibrated with 5% CO_2 in air, pH 8.0, (m-KRB).

The ova were pretreated with 0.01% hyaluronidase (Sigma; type I) solution in m-KRB for 15 min to disperse cumulus cells under 5% CO_2 in air at 37° and the ova from 5—7 females were mixed well to avoid the individual heterogenity. Ova freed from cumulus cells were divided in 5—7 parts and washed twice with m-KRP or m-KRB. Rinsed ova were placed in the chamber (capacity 200 μl) filled with 50 μg/ml of chymotrypsin (Sigma; type II) solution in m-KRP or m-KRB. The zona solubilization was observed continuously at 37° under the phase contrast microscope (Nikon, type MD with incubator). In the experiment shown in Fig. 1, the ova having zona not to be affected by chymotrypsin were collected from the chamber and the zona solubility properties were checked again by putting them into another chamber with freshly prepared enzyme solution.

The zona solubility properties in various concentrations of chymotrypsin (6.25, 12.5, 25 and 50 μg/ml) were examined in m-KRB, pH 8.0, in the same way as described above.

For the experiment of comparative zona solubilizing activities of proteolytic enzyme, trypsin (Sigma; type I), Pronase-P (Kaken Kagaku) and chymotrypsin were used at the concentration of 25 and 50 μg/ml respectively in m-KRB. The ova were subjected for the experiment without hyaluronidase pretreatment in this experiment to examine the effect of such procedure.

The Relationship between the Fertilizing Ability and the Zona Solubility——Superovulated ova were collected in m-KRB at 12, 15, 18, 21 and 24 hr after HCG injection. The ova from the one side of the oviduct were subjected to the experiments of zona solubility properties in m-KRB, and the ova from the other side were subjected to the experiments of *in vitro* fertilization.

In vitro fertilization was done in the same manner as described previously.[12] The ova surrounded with cumulus mass were washed twice with 5 ml of m-KRB and transfered to 0.4 ml of medium covered with paraffin oil. Cauda epididymis from ddy male mouse weighing about 40 g were chopped in 2 ml of m-KRB and 3 min later the upper part (1 ml) of the medium was sucked with Pasteur pipette to make it sperm suspension ($1.0—1.5 \times 10^6$ sperm/ml). Fifty μl of epididymal sperm suspension was inseminated to ova in 0.4 ml of m-KRB. After 120 min incubation under 5% CO_2 in air at 37°, the ova were treated with 0.01% of hyaluronidase solution in m-KRB and the evidence of fertilization was observed under the phase contrast microscope. The ova having sperm in perivitelline space or sperm attached to vitellus and forming enlarged head were judged as fertilized. In this condition the pronuclei formation needed longer period.[12]

To compare the change of zona pellucida *in vitro* with the change in oviduct, the ova collected from the oviduct at 12 hr after HCG injection were cultured *in vitro* for 3, 6, 9 and 12 hr in 0.4 ml of m-KRB at 37° under 5% CO_2 in air, and the zona solubility properties were examined along with the fertilizing ability.

The effects of hormonal treatment on the change of the zona pellucida of ova in oviduct were also studied by injecting additional PMS (5 IU) or HCG (5 IU) at the time of ovulation (12 hr after HCG injection for superovulation). In the cases that ova were collected at 9 and 12 hr after ovulation, the additional injections of gonadotropin were done twice (12 and 18 hr after HCG injection for superovulation).

The Effects of Sperm and the Supernatant of Sperm Suspension on the Zona Solubility Properties——The supernatants of epididymal sperm suspension in m-KRB were prepared by centrifugation (1500 rpm × 10 min) of sperm suspension (1.5×10^6 sperm/ml) followed by filtration with a millipore filter (HAWP 02500; 0.45 μ) to avoid a little sperm swimming up to supernatant fraction. The ova collected at 16 hr after HCG injection

13) T. Iwamatsu and M.C. Chang, *J. Reprod. Fert.*, **26**, 197 (1971).

were put into this (or 10 fold diluted with medium) supernatant of sperm suspension. After 2 hr incubation the ova were transfered to chymotrypsin solution and the zona solubility properties were examined.

The change of the zona solubility properties after insemination of capacitated or epididymal spermatozoa was observed by time wise. Epididymal sperm suspension was used within 5 min after preparation and capacitated sperm were obtained by preincubation of epididymal sperm in m-KRB for 60 min. Washed ova in cumulus mass collected at 16 hr after HCG injection were transfered to epididymal or capacitated sperm suspension ($1.0-1.5\times10^5$ sperm/ml) in the chamber (200 μl) which was described before and incubated for various time at 37°. At the end of the incubation 40 μl of enzyme solution in m-KRB (chymotrypsin; 250 μg/ml, hyaluronidase; 1 mg/ml) was added to the chamber and the ratio of zonae showing resistivity to chymotrypsin was examined.

Results

The zona solubility properties to chymotrypsin were investigated in phosphate buffered medium and bicarbonate buffered medium. In two kinds of medium the zona solubility properties of ova found to be different (Fig. 1).

The enzymatic treatment of superovulated ova collected at 12 hr after HCG injection caused the dissolution of zona pellucida of all the ova within 10 min in both medium (A).

When the ova collected at 18 and 24 hr after HCG injection were treated in m-KRP, the time required for zona dissolution extended but all of the ova lost their zona pellucida within 90 min. In m-KRB, on the contrary, the zona pellucida of some ova dissolved within 10 min but the remainder showed no change at least within 90 min incubation (B) (C). The ova having unaffected zona pellucida at 60 min incubation in m-KRB were collected in less than 5 μl of droplet with a finely drawned pipette and transfered to freshly prepared enzyme solution. As shown in (D), the zona pellucidae were not dissolved any more in m-KRB containing chymotrypsin. However, in m-KRP containing chymotrypsin they were dissolved. The time required for removal of zona pellucida was resembled to that of fresh ova treated in m-KRP. Since the successes of fertilization *in vitro* were reported only in bicarbonate buffered medium,[2),8),9),13)] the nature of zona solubility properties in m-KRB was further investigated.

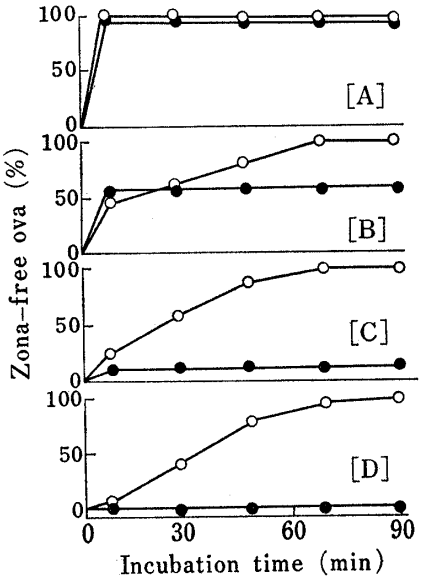

Fig. 1. Dissolution of Zona Pellucida with Chymotrypsin in m-KRP and m-KRB Medium

Ova were treated with chymotrypsin (50 μg/ml) in each medium. m-KRP, m-KRB; see text. Ova collected from superovulated female at 12, 18 and 24 hr after HCG injection were subjected to the experiment [A], [B] and [C] respectively. In [D] the ova having undissolved zona pellucida after 60 min incubation in m-KRB medium were transfered to freshly prepared enzyme solution in m-KRP or m-KRB.
—○—: m-KRP, —●—: m-KRB.

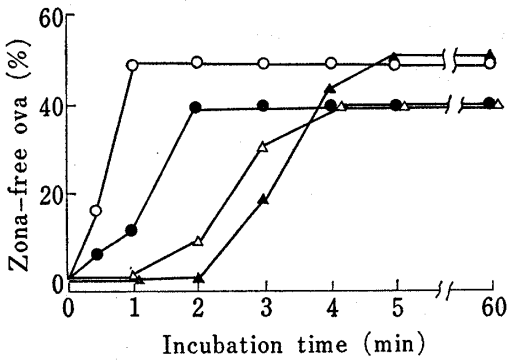

Fig. 2. Disappearance of Zona Pellucida from Ova incubated with Various Concentrations of Chymotrypsin in m-KRB

—○—: 50 μg/ml, —●—: 25 μg/ml, —△—: 12.5 μg/ml, —▲—: 6.25 μg/ml.
ova: 20 hr after HCG injection.

The final ratio of ova having resistivity to chymotrypsin in m-KRB was not affected by the concentration of the enzyme solution, while the time required for dissolution took longer period when treated with low concentration of enzyme solution (Fig. 2).

Comparative zona solubilizing activities of proteolytic enzyme were examined between trypsin, Pronase-P and chymotrypsin at the concentration of 25 and 50 μg/ml respectively. To distinguish the solubility activity clearly, ova collected from the oviduct at 20 hr after HCG injection were subjected for the experiment that about the half of the ova lost the susceptibility to chymotrypsin. After 60 min incubation the ratio of zona-free ova was examined. There was no difference in zona solubilizing activity between these enzymes. To investigate whether the hyaluronidase have an effect on the zona solubilizing activity or not, under the presence of hyaluronidase, zona solubility properties to chymotrypsin were also examined. Similar portion of ova lost their zona pellucida with chymotrypsin in the presence of hyaluronidase (Table I).

Table I. Dissolution of Zona Pellucida with Several Proteases in m-KRB Medium

Enzyme	Dose (μg/ml)	Total no. of ova examined	No. of ova denuded	Zona-free ova (%)
Chymotrypsin	50	33	17	52
	25	29	14	48
Trypsin	50	35	17	49
	25	38	17	45
Pronase-P	50	30	15	50
	25	25	11	44
Chymotrypsin +hyaluronidase	25 +100	67	34	51

Ova collected 20 hr after HCG injection were incubated in each enzyme solution for 60 min.

These results suggest that there are two types of zona pellucida, one being dissolved by the treatment of proteases while the other being resistant to it. So the relation between such characteristics of zona pellucida and the fertilizing ability of ova was investigated. The results are summarized in Table II. Fertilization potency of mouse ova decreased gradually with lapse of time after ovulation and almost disappeared at 24 hr after application of HCG. Sensitivity of zona pellucida to chymotrypsin decreased in the way quite similar to it. Such loss of fertilization potency did not occur on the ova taken from the oviduct at 12 hr after

Table II. The Relationship between Fertilizing Ability of Mouse Ova and the Sensitivity of Zona Pellucida to Chymotrypsin

Time after HCG injection (hr)	Ova from oviduct[a]						Ova cultured *in vitro*[b]	
	Superovulated		Superovulated +HCG treated		Superovulated +PMS treated			
	ZFO[c]	fert.[d]	ZFO	fert.	ZFO	fert.	ZFO	fert.
12	82± 7	90± 6	—	—	—	—	—	—
15	92± 4	78± 4	90± 6	82± 8	96± 6	80±5	93± 5	86±10
18	72± 9	54± 7	48±12	46±13	94± 5	75±5	76± 7	74±10
21	46±15	24±10	25± 7	19± 6	79±11[e]	68±7[e]	82±13[e]	69± 6[e]
24	12±10	4± 2	34±10	16± 5	81± 8[e]	52±8[e]	81±14[e]	84± 8[e]

All values represent mean±s.e.

a) Ova were collected from superovulated female oviduct at various time after ovulation.

b) Ova collected from superovulated female oviduct at 12 hr after HCG injection and cultured *in vitro* for 3, 6, 9 and 12 hr were expressed as 15, 18, 21 and 24 hr after HCG injection respectively.

c) Percentage of zona-free ova; Ova were incubated in chymotrypsin solution (25 μg/ml of m-KRB medium) for 60 min.

d) Percentage of ova with sperm in perivitelline space or with enlarged sperm head.

e) $p<0.05$; significantly different from "superovulated" in each time after HCG injection.

HCG injection and cultured in m-KRB for 3 to 12 hr. Simultaneously, the sensitivity of zona pellucida to chymotrypsin was also maintained in such ova.

The additional injection of PMS to superovulated female at 12 hr (or 12 and 18 hr) after HCG injection prevented the loss of the fertilization potency of ova and the sensitivity of zona pellucida to chymotrypsin in oviduct. Such effect was not observed with HCG treatment.

Apart from the change of ova in oviduct, the sensitivity of zona pellucida to chymotrypsin decreased significantly in *in vitro* fertilized ova. Such change was not caused by the supernatants of sperm suspension (Table III).

Table III. Effects of Sperm and Supernatant of Sperm Suspension on the Sensitivity of Zona Pellucida to Chymotrypsin

Treatment	Total no. of ova examined	No. of experiments	Zona-free ova (%)
None	133	4	$83 \pm 9^{a)}$
1.5×10^5 sperm suspension	103	4	$5 \pm 3^{a,b)}$
Sup of 1.5×10^5 sperm suspension	68	2	71
Sup of 1.5×10^6 sperm suspension	56	2	75

a) Mean±s.e. Ova (16 hr after HCG injection) were preincubated with or without sperm for 2 hr before incubation with chymotrypsin.
b) $p<0.01$.

These changes of zona pellucida, which normally require several hours in the oviduct, proceeded in short time when ova were inseminated to sperm suspension. Besides, there was an evident difference in time to cause changes on zona between the case of epididymal sperm and capacitated sperm were used. The loss of dissolution susceptibility in these treatments was associated with the time of sperm penetration reported in previous paper[2] (Table IV).

Table IV. Change in Sensitivity of Zona Pellucida to Chymotrypsin after Insemination of Spermatozoa

	No. of zona-free ova/no. of ova examined (%)									
	Time after insemination (min)									
	5	10	15	20	25	30	35	40	45	50
Epididymal Spermatozoa	—	22/22 (100)	30/30 (100)	29/31 (94)	20/28 (71)	29/35 (84)	20/28 (71)	19/25 (76)	2/19 (11)	1/29 (3)
Capacitated Spermatozoa[a)]	20/21 (95)	23/35 (66)	0/38 (0)	14/53 (26)	2/30 (7)	3/34 (9)	3/39 (8)	0/46 (0)	2/38 (6)	—

a) Epididymal spermatozoa were preincubated in modified-KRB buffer for 60 min. ova; 16 hr after HCG injection.

Discussion

Braden, *et al.*[10] have shown that the property of zona pellucida of mammalian ova changes after fertilization so that the penetration of further spermatozoa is greatly reduced (zona reaction). It is presumed that the zona reaction is the result of interaction between zona pellucida and the cortical granule materials.[14] Besides the prevention of further sperm penetration, there are many reports which suggest the change of biological and physicochemical

14) C.R. Austin and A.W.H. Braden, *J. Exp. Biol.*, **33**, 358 (1956); C. Baross and R. Yanagimachi, *Nature, London*, **233**, 268 (1971).

properties on zona pellucida associated with zona reaction.[11a,d,g,15] Concerning with the solubility properties of zona, surrounding fertilized and unfertilized ova, many solubilizing agents or conditions were investigated, such as 2-mercaptoethanol, sodium periodate, proteases or acidic solutions.[11b,c,e,f] Generally, these authors clarified the zona pellucida of unfertilized ova were consistently more readily dissolved than those of fertilized ova. On the other hand, Krzanowska[12] reported the difference of the zona solubility properties to trypsin between the unfertilized ova collected shortly after ovulation and the old unfertilized ova in oviduct. However, the difference detected between them was the change of time required for removal of zona pellucida. In this report, it was shown that the zona pellucida of unfertilized ova is present in one of two forms, one of which is sensitive to a range of proteases while the another type is totally unaffected by such enzymatic treatment in m-KRB. Concerning with the fact that the structure of zona may change in different buffered medium, the zona solubility properties to proteases may depend on its environments.

Since, the occurrence of fertilization is observed in individual ovum as fertilized or not and the successes of *in vitro* fertilization were only reported in bicarbonate buffered medium, the change in time required for removal of zona pellucida by proteases in KRP could not directly combined with the fertilizing ability of ova. However, the all or none zona dissolution in m-KRB observed in present paper explained clearly the change of fertilizing ability of ova in oviduct or that of after fertilization.

Susceptibility of zona removal by proteases diminished as the time between ovulation and recovery increased, and the proportion of ova fertilizable *in vitro* was of the same order and followed the same pattern. This suggests that the change of zona leads to the prevention of sperm penetration into zona pellucida by its trypsin-like enzyme. It can be said that a part of ova used for *in vitro* fertilization has no fertilizing ability at the start of the fertilization procedure.

As shown in Table IV, zona reaction also caused the disappearance of sensitivity of zona pellucida to chymotrypsin. Besides the participation of spermatozoa, the similarity of these two cases of zona alteration (the ova became unfertilizable and the zona became insoluble by proteases) offered the possibility of the same phenomenon occurred on zona pellucida. The changes of zona solubility properties were prevented by an additional injection of PMS or a cultivation of ova *in vitro*. This suggests clearly that the change of zona solubility properties in oviduct, which may be termed self-zona reaction, is influenced by the surroundings of ova. Although the chemical nature of the factors, which serve to the change of zona pellucida in oviduct, must await further investigation, the fact that the changes with time in zona susceptibility to protease and the fertilizing ability are related to the effect of PMS on oviduct function is very interesting matter.

15) R.B.L. Gwatkin, D.T. Williams, J.F. Hartmann, and M. Kuizuk, *J. Reprod. Fert.*, 32, 259 (1973).