TRYPSIN-MEDIATED MODIFICATION OF THE ZONA PELLUCIDA GLYCOPEPTIDE STRUCTURE OF HAMSTER EGGS

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One of the mechanisms that prevents polyspermy in mammals is the zona reaction, which involves a modification of the zona pellucida (Braden, Austin & David, 1954; Austin, 1961). Cortical granules have been implicated in this process because upon stimulation or fertilization of the egg they release a substance(s) which acts on the zona and renders it impenetrable to spermatozoa (Austin & Braden, 1956; Barros & Yanagimachi, 1971). Gwatkin, Williams, Hartmann & Kniazuk (1973) have proposed that the zona reaction is mediated by a trypsin-like protease released from the cortical granules which attacks the zona sperm-receptors. Vacquier, Tegner & Epel (1972, 1973) have proposed a similar mechanism for the block to polyspermy in invertebrate eggs. We report here that low concentrations of crystalline trypsin modify the glycopeptide structure of the zona pellucida, and this modification is detected by plant lectins.

Mature unfertilized eggs of the golden hamster were obtained as follows. Adult hamster females were each injected intraperitoneally with 20 to 30 i.u. PMSG in the morning (09.00 to 11.00 hours) of Day 1 (day of ovulation) of the oestrous cycle followed by an intraperitoneal injection of 20 to 30 i.u. HCG in the afternoon or evening (15.00 to 22.00 hours) of Day 3 of the oestrous cycle. Between 15 and 17 hr after injection of HCG, the animals were killed and their oviducts were flushed with Tyrode's solution containing 0.1% crystalline bovine serum albumin (Reheis Chem. Co., Chicago, U.S.A.) (TA). The eggs were freed from the surrounding cumulus cells by treatment for 15 min at 25°C with 0.1% bovine testicular hyaluronidase (300 USP units per mg; Nutritional Biochem. Corp., Cleveland, U.S.A.) in TA. To obtain fertilized eggs at the pronuclear stage, females were treated with PMSG and HCG as above. About 7 hr after injection of HCG, spermatozoa from the cauda epididymidis were suspended in TA and inseminated directly into the uterus. The oviducts were flushed with TA 16 hr later. Treatment of fertilized eggs with hyaluronidase was unnecessary since the cumulus cells had dispersed by the time of recovery. Unfertilized and fertilized eggs were rinsed with TA and each type was separated into two groups. The eggs in one group of each type were treated for 5 to 120 min at 25°C with 0.0001% or 0.001% bovine pancreatic trypsin (twice crystallized, Type III,

10,000 to 13,000 BAEE units/mg; Sigma Chem. Co., Saint Louis, U.S.A.) in 0.01 M-tris-HCl:0.9% NaCl:0.1% crystalline albumin (pH 7.4) containing 100 µg/ml affinity purified wheat germ agglutinin (WGA) (Burger & Goldberg, 1967), or with *Ricinus communis* agglutinin with a molecular weight of 120,000 (RCAI) (Nicolson & Blaustein, 1972), or with *Dolichos biflorus* agglutinin (DBA) (Etzler & Kabat, 1970). After incubation for 30 min in one of the lectin solutions at 25°C, the eggs were tested for (a) the ability to agglutinate through their zonae pellucidae and (b) changes in the light-scattering properties of their zonae pellucidae (Oikawa, Yanagimachi & Nicolson, 1973; Oikawa, Nicolson & Yanagimachi, 1974). For the agglutination experiments, the eggs in a lectin solution were aggregated by pushing about ten of them together with a needle. The dishes were then agitated to disperse the aggregates. Agglutination was qualitatively scored + + + when all the eggs remained firmly attached through their zonae despite repeated agitation (Pl. 1, Fig. 1), + + when the eggs dispersed into two or three aggregates of three to six eggs each, + to ± when the aggregates contained only two to three eggs each, and – when the eggs completely dispersed (Pl. 1, Fig. 2). Light scattering of the zona pellucida was scored + when the outermost surface of the zona scattered light strongly under dark-field illumination (Pl. 1, Fig. 3), and ± or – when there was little or no light scattering (Pl. 1, Fig. 4) (Oikawa *et al.*, 1973, 1974). The other group of eggs (control) was treated in the same manner except for the omission of the trypsin pretreatment or for the inclusion of lectin saccharide inhibitors in the lectin solutions.

In another series of experiments, the ability of trypsin pretreatment to render the zona pellucida of unfertilized eggs impenetrable to spermatozoa was examined. The unfertilized eggs (freed from the cumulus cells) were treated for 5 to 120 min with 0.0001% or 0.001% trypsin as described above. The eggs were thoroughly rinsed with TA and exposed *in vitro* to capacitated spermatozoa which had undergone an acrosome reaction (Oikawa *et al.*, 1973, 1974). Two hours later, the eggs were examined with a phase-contrast microscope for sperm attachment and evidence of sperm penetration into or through the zona pellucida. The eggs (zonae) were recorded as unpenetrated if spermatozoa were attached to the surface but none was within or through the zona.

When hamster eggs were treated with lectins without pretreatment with trypsin, they agglutinated strongly (RCAI), moderately (DBA) or not at all (WGA) (Table 1, Exps 1 and 8). Eggs treated with lectin showed strong light scattering from the zona surface (Table 1, Exps 1 and 8). These lectin-induced properties were specific because inclusion of the appropriate saccharide inhibitors, e.g. 0.1 M-lactose for RCAI, 0.1 M-N-acetyl-D-galactosamine for DBA and 0.1 M-N-acetyl-D-glucosamine for WGA, abolished these effects. Pretreatment of unfertilized eggs with trypsin inhibited sperm penetration of the zona (Table 1, Exps 2 to 5 and 7). It also increased the ability of eggs to agglutinate with WGA, but not with RCAI or DBA (Table 1, Exps 5 and 7). The trypsin-induced increase in agglutination with WGA could be blocked by the addition of soybean trypsin inhibitor (Type I-S, Sigma Chemical Co., Saint Louis, U.S.A.) (Table 1, Exp. 6). Pretreatment with trypsin also prevented lectins from inducing light scattering from the zona (Table 1, Exps 4, 5, 7 and 9).

PLATE 1

FIG. 1. Unfertilized hamster eggs strongly agglutinated (+++) by RCAI. $\times 130$.

FIG. 2. Unfertilized hamster eggs that failed to agglutinate by RCAI in the presence of a saccharide inhibitor, 0.1 M-lactose. $\times 130$.

FIG. 3. Strong light scattering (+) of the outermost region of the zona pellucida of a hamster egg treated with RCAI. Dark-field, $\times 420$.

FIG. 4. Absence of light scattering of the outer region of the zona pellucida when a hamster egg was treated with RCAI in the presence of a saccharide inhibitor, 0.1 M-lactose. Dark-field, $\times 420$.

Table 1. Effects of trypsin on lectin-induced hamster egg agglutination and light scattering from the zonae

Eggs	Exp. no.	Pretreatment		% Zonae penetrated by sperm†	Response of zona to lectin‡					
					WGA		RCAI		DBA	
		Trypsin*	SBTI	Time (min)	Agglutination	Light scatter	Agglutination	Light scatter	Agglutination	Light scatter
Unfertilized	1	0	0	0	—	+	+	+	+	+
	2	0.001%	0	5	—	+	+	+	+	±
	3	0.001%	0	10	—	+	+	+	+	—
	4	0.001%	0	15-20	± to +	+	+	+	+	—
	5	0.001%	0	25	+	+	+	+	+	—
	6	0.001%	0.001%	25	—	+	+	+	+	+
	7	0.0001%	0	120	+	—	+	+	+	—
Fertilized	8	0	0	0	—	+	+	+	+	+
	9	0.001%	0	25	+	—	+	+	+	—

SBTI, soybean trypsin inhibitor; WGA, wheat germ agglutinin; RCAI, *Ricinus communis* agglutinin; DBA, *Dolichos biflorus* agglutinin.

* Bovine pancreatic trypsin.

† At least twenty-five to thirty eggs in each group, checked 2 hr after insemination.

‡ See text for details of procedure and scoring.

In contrast to the effects of trypsin, fertilization of hamster eggs *in vivo* did not result in an increase in egg agglutination with WGA or prevent lectin from inducing light scattering, but it did block sperm penetration through the zona (Table 1, Exp. 8).

The results indicate that trypsin treatment such as that employed by Hartmann & Gwatkin (1971), e.g. 0.0001% for 120 min at 25°C; Table 1, Exp. 7, results in zona modifications that are not found after fertilization *in vivo*. Thus, the zona reaction mediated by fertilization may be different from the block to fertilization induced by trypsin. These experiments do not rule out the possibility that very brief trypsin treatment (see Table 1, Exp. 2) may actually mimic the native zona reaction. The possibility also exists that fertilization results in the release of other non-protease enzymes (such as glycosidase or glucanase) similar to those found by Epel, Weaver, Muchmore & Schimke (1969) in sea urchin eggs. Under physiological conditions, protease(s), and possibly other enzymes such as glycosidases, may jointly alter the zona pellucida to prevent sperm binding and penetration. The blocking of sperm penetration by purified trypsin alone may be due to proteolytic destruction of the zona pellucida, and may not reflect the natural mechanism of the zona reaction.

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