

Prevention of fertilization by exposure of hamster eggs to soluble acrosin

R. B. L. Gwatkin, L. Wudl*, E. F. Hartree† and E. Fink‡

Merck Institute for Therapeutic Research, Rahway, New Jersey, U.S.A.;

†A.R.C. Institute of Animal Physiology, Animal Research Station, Cambridge, U.K. and

‡Institute of Clinical Chemistry, University of Munich, Munich, West Germany

Incubation of hamster eggs with trypsin or chymotrypsin, but not with a series of glycosidases or lipases, prevents capacitated spermatozoa from binding to the zona pellucida so that fertilization is prevented (Hartmann & Gwatkin, 1971; Gwatkin, Williams & Andersen, 1973). Inactivation of the binding sites for spermatozoa on the zona pellucida by a trypsin-like protease released by discharge of cortical granules also appears to be responsible for the zona reaction which prevents polyspermy (Gwatkin, Williams, Hartmann & Kniazuk, 1973). Acrosin is also a trypsin-like protease and it can be extracted from sperm acrosomes. We therefore investigated whether the fertilization of hamster eggs is blocked by incubation with soluble acrosin.

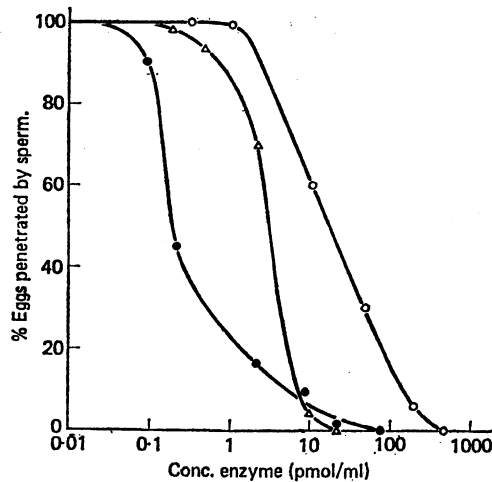
Eggs were collected from hamsters induced to superovulate and the cumuli oophori were removed with hyaluronidase as described previously (Gwatkin, Andersen & Hutchison, 1972). Groups of such cumulus-free eggs were incubated under oil in 20- μ l drops of PBS (pH 7.3; Dulbecco & Vogt, 1954) containing various concentrations of trypsin or acrosin for 2 h at 37.5°C on a rocker (5–6 oscillations/min). The eggs were then washed in medium 199M2 (Gwatkin & Andersen, 1973) and added to 40- μ l drops of a suspension of epididymal spermatozoa previously capacitated by incubation for 5 h in medium 199M2 containing cumulus cells (Gwatkin *et al.*, 1972). After incubation for an additional 90 min, the number of spermatozoa bound to the eggs and the penetration of eggs by spermatozoa were recorded under a phase-contrast microscope.

Preliminary experiments were carried out with purified boar acrosin (Fritz *et al.*, 1975). At a protein concentration of approximately 10 μ g (240 pmol acrosin)/ml, this enzyme completely inhibited sperm binding and penetration. In the remaining experiments, a range of molar concentrations of hamster and ram acrosin was used. These enzymes also blocked sperm binding, but for convenience only penetration was determined quantitatively.

The molar concentration of the enzyme preparations was determined by fluorometric titration using 4-methylumbelliferyl-*p*-guanidinobenzoate (Brown, Andani & Hartree, 1975a). Soluble ram acrosin was isolated from washed ejaculated ram spermatozoa as the fraction 'FD' of Brown *et al.* (1975b). This material was subjected to affinity chromatography on a column of Sepharose-4B linked to 4(4'-aminophenoxypropoxy)benzamidine (Jameson & Elmore, 1974). Acrosin was obtained as a single protein that contained 12 nmol enzyme/mg. Soluble hamster acrosin was obtained by acid extraction of washed epididymal spermatozoa, followed by chromatography of the extract, first on Sephadex G-100 and then on *p*-benzamidine linked with a spacer arm to an insoluble matrix, according to the procedures of Fritz *et al.* (1975). The hamster acrosin preparation contained 0.52 nmol enzyme/mg. The activities of these acrosin preparations were compared with that of bovine pancreatic trypsin (crystalline: Worthington Freehold), which contained 19 nmol enzyme/mg. The enzymes were dissolved in 0.5 mM-HCl and kept at 5°C overnight before being diluted to the required concentrations.

As shown in Text-fig. 1 pretreatment of eggs with pancreatic trypsin for 2 h completely suppressed sperm penetration, confirming earlier results (Hartmann & Gwatkin, 1971): an inhibition of 50% was obtained with 0.2 pmol trypsin/ml. The acrosin preparations also suppressed sperm penetration, although higher enzyme concentrations were required and the dose-response curves were different. For 50% inhibition, 7 pmol hamster acrosin/ml and 20 pmol ram acrosin/ml were required. However, the amount of protease available to the eggs may have been lower than these values

* Present address: Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey, U.S.A.



Text-fig. 1. Effect of pretreating hamster eggs with various concentrations of bovine pancreatic trypsin (●) and hamster (△) or ram (○) acrosin on penetration by capacitated hamster spermatozoa. Each point is derived from 30 eggs.

because of adsorption onto glass, plastic or oil interfaces. The greater activity of the hamster acrosin could have been due to a species specificity or to proteolytic contamination in this relatively impure preparation.

Although the evidence is not conclusive (Miyamoto & Chang, 1973; Gwatkin, 1976), acrosin is believed to be the enzyme responsible for zona penetration by the spermatozoon (Stambaugh & Buckley, 1969; Stambaugh, Brackett & Mastroianni, 1969; Zaneveld, Robertson, Kessler & Williams, 1971). Acrosin is active in a bound form, probably as a peripheral protein of the inner acrosomal membrane, the soluble form being an artefact of the isolation procedure (Brown *et al.*, 1975b). The sharply defined penetration channel observed in the zona pellucida (Bedford, 1968; Gwatkin, Carter & Patterson, 1976) is consistent with the action of a membrane bound enzyme. Because pretreatment of hamster eggs with soluble acrosin prevents fertilization *in vitro* (present results), any accidental liberation of this form of the enzyme in the vicinity of the eggs *in vivo* would be potentially contraceptive. Such an accident is either rare or counteracted by inhibitors in the oviductal fluid (Stambaugh, Seitz & Mastroianni, 1974). Inhibitors isolated from seminal plasma affect the soluble but not the bound form of ram acrosin (Brown & Hartree, 1976).

Soluble acrosin closely resembles pancreatic trypsin (Fritz *et al.*, 1975) and their actions on the sperm binding sites of the zona pellucida are probably similar. Trypsin and soluble rabbit acrosin also have similar effects on erythrocytes, removing agglutination-site glycopeptides (Uhlenbruck, Sprenger, Schumacher & Zaneveld, 1972). These proteases, as well as the enzyme discharged by the cortical granules, presumably hydrolyse arginyl and lysyl bonds in the zona pellucida, thus altering the conformation of the receptor so that it cannot bind spermatozoa (Gwatkin & Williams, 1977). Repin & Akimova (1976) have reported that the zona reaction in mouse eggs is followed by a partial hydrolysis of the zona.

We thank Mr O. F. Andersen for skilled technical assistance and Dr W.-D. Schleuning for a gift of boar acrosin.

References

- BEDFORD, J.M. (1968) Ultrastructural changes in the sperm head during fertilization in the rabbit. *Am. J. Anat.* **123**, 329-358.
- BROWN, C.R. & HARTREE, E.F. (1976) Effects of acrosin inhibitors on the soluble and membrane-bound forms of ram acrosin, and a reappraisal of

- the role of the enzyme in fertilization. *Z. Physiol. Chem.* **357**, 57-65.
- BROWN, C.R., ANDANI, Z. & HARTREE, E.F. (1975a) Studies on ram acrosin: fluorimetric titration of operational molarity with 4-methylumbelliferyl-p-guanidinobenzoate. *Biochem. J.* **149**, 147-154.
- BROWN, C.R., ANDANI, Z. & HARTREE, E.F. (1975b) Studies on raw acrosin: isolation from spermatozoa, activation by cations and organic solvents, and influence of cations on its reaction with inhibitors. *Biochem. J.* **149**, 133-146.
- DULBECCO, R. & VOGT, M. (1954) Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.* **199**, 167-182.
- FRITZ, H., SCHLEUNING, W.-D., SCHIESSLER, H., SCHILL, W.-B., WENDT, V. & WINKLER, G. (1975) Boar, bull and human sperm acrosin.—Isolation, properties and biological aspects. In *Proteases and Biological Control*, pp. 715-735. Eds E. Reich, D. Rifkin & E. Shaw. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- GWATKIN, R.B.L. (1976) Fertilization. In *The Cell Surface in Animal Development*, Ch. 1, pp. 1-54. Eds G. Poste & G.L. Nicolson. North-Holland, Amsterdam.
- GWATKIN, R.B.L. & ANDERSEN, O.F. (1973) Effect of glycosidase inhibitors on the capacitation of hamster spermatozoa by cumulus cells *in vitro*. *J. Reprod. Fert.* **35**, 565-567.
- GWATKIN, R.B.L. & WILLIAMS, D.T. (1977) Receptor activity of the hamster and mouse solubilized zona pellucida before and after the zona reaction. *J. Reprod. Fert.* **49**, 55-59.
- GWATKIN, R.B.L., ANDERSEN, O.F. & HUTCHISON, C.F. (1972) Capacitation of hamster spermatozoa *in vitro*: the role of cumulus components. *J. Reprod. Fert.* **30**, 389-394.
- GWATKIN, R.B.L., WILLIAMS, D.T. & ANDERSEN, O.F. (1973) Zona reaction of mammalian eggs: properties of the cortical granule protease (cortin) and its receptor substrate in hamster eggs. *J. Cell Biol.* **59**, 128a.
- GWATKIN, R.B.L., WILLIAMS, D.T., HARTMANN, J.F. & KNIAZUK, M. (1973) The zona reaction of hamster and mouse eggs: production *in vitro* by a trypsin-like protease from cortical granules. *J. Reprod. Fert.* **32**, 259-265.
- GWATKIN, R.B.L., CARTER, H.W. & PATTERSON, H. (1976) Association of mammalian sperm with cumulus cells and the zona pellucida studied by scanning electron microscopy. In *Scanning Electron Microscopy/1976*, Vol. 2, Pt. 6, pp. 379-384. Eds H. Johari & R. P. Decker. IIT Res. Inst., Chicago, Illinois.
- HARTMANN, J.F. & GWATKIN, R.B.L. (1971) Alteration of sites on the mammalian sperm surface following capacitation. *Nature, Lond.* **234**, 479-481.
- JAMESON, G.W. & ELMORE, D.T. (1974) Affinity chromatography of bovine trypsin. A rapid separation of bovine α - and β -trypsin. *Biochem. J.* **141**, 555-565.
- MIYAMOTO, H. & CHANG, M.C. (1973) Effects of protease inhibitors on the fertilizing capacity of hamster spermatozoa. *Biol. Reprod.* **9**, 533-537.
- REPIN, V.S. & AKIMOVA, I.M. (1976) The microelectrophoretic analysis of protein patterns of mammalian oocyte and zygote zonae pellucidae. *Biokimia (U.S.S.R.)* **41**, 50-57.
- STAMBAUGH, R. & BUCKLEY, J. (1969) Identification and subcellular localization of the enzymes effecting penetration of the zona pellucida by rabbit spermatozoa. *J. Reprod. Fert.* **19**, 423-432.
- STAMBAUGH, R., BRACKETT, B.G. & MASTROIANNI, L. (1969) Inhibition of *in vitro* fertilization of rabbit ova by trypsin inhibitors. *Biol. Reprod.* **1**, 223-227.
- STAMBAUGH, R., SEITZ, H.M. & MASTROIANNI, L. (1974) Acrosomal proteinase inhibitors in rhesus monkey (*Macaca mulatta*) oviduct fluid. *Fert. Steril.* **25**, 352-357.
- UHLBRUCK, G., SPRENGER, I., SCHUMACHER, G.F.B. & ZANEVELD, L.J.D. (1972) Additional properties of acrosin, a proteolytic enzyme from rabbit sperm acrosomes. *Naturwissenschaften* **59**, 124-125.
- ZANEVELD, L.J.D., ROBERTSON, R.T., KESSLER, M. & WILLIAMS, W.L. (1971) Inhibition of fertilization *in vivo* by pancreatic and seminal plasma trypsin inhibitors. *J. Reprod. Fert.* **25**, 387-392.

Received 13 December 1976