BIOLOGY OF REPRODUCTION 15, 102–106 (1976)

Involvement of Esterases in Sperm Penetration of the Corona Radiata of the Ovum

M. M. BRADFORD, R. A. McRORIE and WILLIAM L. WILLIAMS

Reproduction Research Laboratories,
Department of Biochemistry,
University of Georgia,
Athens, Georgia 30602

Downloaded from https://academic.oup.com/biolreprod/article/15/1/102/2841675 by guest on 21 August 2022

ABSTRACT

During fertilization the spermatozoon must penetrate several layers surrounding the ovum prior to actual gamete fusion. It has been previously reported that acrosomal extracts of rabbit spermatozoa will disperse the cumulus layer, the corona radiata, and the zona pellucida from rabbit ova. The data presented here demonstrate that the esterolytic activity present in sperm acrosomes is involved in the dispersal of the corona radiata and thus in the penetration of the intracellular cement of the corona radiata during the fertilization process.

Cumulus free ova have been shown to be subject to dispersal of corona radiata cells by the action of alkaline buffers. This in conjunction with the observation that the corona is dispersed by esterolytic activity is indicative of an ester linkage functioning in the macromolecular cementing substance in the intracellular matrix of the corona radiata of rabbit ova.

INTRODUCTION

The follicular cell mass of the rabbit ovum consists of two distinct layers, the cumulus cells and the corona radiata. The cumulus cells are dispersed by hyaluronidase (Austin, 1948; Chang, 1950) while the corona radiata cells are dispersed by an enzyme tentatively designated the corona penetrating enzyme (Zaneveld et al., 1969; Zaneveld and Williams, 1970).

In very early work with crude preparations, Pincus and Enzmann (1932, 1936) reported the presence of a substance in sperm that dispersed the follicular cells around the ovum. Yamane (1935a, b) treated rabbit sperm with aqueous toluene obtaining an extract which dispersed both the cumulus and the corona cells and dissolved the zona pellucida of rabbit ova. Such an activity was first reported to be localized in acrosomes by Srivastava et al. (1965a, b). Preliminary characterization of corona penetrating enzyme activity was reported by Zaneveld et al. (1969), Zaneveld and Williams (1970), Tillman (1972), Bradford and Lehnhardt (1974), and Bradford (1974, 1975); however, the nature of the enzyme and hence the nature of the macromolecular substances binding the corona radiata together was unknown prior to this report.

Accepted March 31, 1976.
Received September 2, 1975.

MATERIALS AND METHODS

The following chemical reagents were obtained from the suppliers indicated. Azocoll (Calbiochem), Sephadex (Pharmacia), Cellex D (Biorad), Gestyl (Pregnant mare's serum; PMS) (Organon), Human Chorionic Gonadotropin (HCG) (Spencer Meade), hyaluronidase (Worthington) Hyamine 2389 (methyl-dodecyl-benzyl-trimethyl-ammonium chloride and methyl-dodecyl-xylene-bis-trimethyl-ammonium chloride) (Rohm and Haas), Triton X-100 (*p*-tert-octyl-phenoxy-polyethoxy-ethanol oligomers) (Schwartz Mann), Concanavalin A-Sepharose (Pharmacia), and *t*-butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester (Cyclo Chemicals). N-acetyl-alanyl-alanyl-prolyl-alanyl-chloromethyl ketone was the gift of Dr. James Travis.

Superovulation Technique

Singly-housed New Zealand White female rabbits were given 100 IU PMS intramuscularly. The PMS treated animals were given 150 IU HCG intravenously 96 h post-PMS administration. The animals were sacrificed 13 h after HCG administration. The ovary, oviduct, and upper third of the uterus were removed, and ova were flushed out the fimbrial end of the oviduct with 3 ml of 50 mM Tris HCl, pH 7.5, buffer. An average of approximately 10 ova per oviduct were obtained with the described procedure.

Assay for Corona Radiata Dispersal

The corona dispersing activity of the substances being tested was measured by determining the degree of removal of the corona radiata from freshly ovulated rabbit ova by essentially the method of Tillman (1972). Ova obtained by the superovulation technique were recovered in cumulus clots which were generally aggregated into one or two large masses each contain-

ing many ova. The cumulus masses were dispersed by incubating for 15–20 min at room temperature in 2 ml of 50 mM Tris HCl, pH 7.5, containing 0.15 M NaCl and 1 mg/ml hyaluronidase (473 N.F. units/mg). All traces of hyaluronidase were removed from the ova by rinsing three times in fresh 50 mM Tris HCl buffer, pH 7.5. Four washed ova were placed into 1 ml pyrex beakers and sealed with Parafilm. Enzyme assays were incubated at 37°C in 50 mM Tris HCl, pH 7.5, in a total volume of 0.4 ml. The ova were examined at intervals using an Olympus inverted microscope to monitor the dispersal of the corona cells.

Preparation of Rabbit Testicular Acetone Powder

Frozen, epididymis-free, rabbit testes were suspended in 1.5 ml cold acetone (−30°C) per g of testicular material and chopped to a fine consistency in a Waring blender at high speed. The solids were removed by vacuum filtration on a Buchner funnel and washed three times with 1.5 ml cold acetone per g of testicular material. The washed material was spread on chromatography paper and dried in air. The dry, acetone-free material was pulverized by spreading with a spatula and stored desiccated at −20°C. No detectable loss of enzyme activity was observed over a 1 year period.

Acrosomal Extraction Procedure

Acrosomal extracts were prepared by the sequential method of Srivastava et al. (1974).

Synthesis of p-nitrophenyl Acetate

The O-acetyl derivative of *p*-nitrophenol was prepared by direct acetylation. The acetylation reaction was performed by the addition of acetic anhydride to a solution of the alcohol in pyridine according to the method of Shriner et al. (1964). Product was precipitated by pouring the reaction mixture into three volumes of ice water and cream-white crystals collected by filtration on a Buchner funnel. The derivative, recrystallized from alcohol and water, exhibited the expected loss of yellow color that reappeared on alkaline hydrolysis.

Assay for Esterase Activity

The esterase hydrolysis of nitrophenyl acetate was monitored by following the increase in absorbance at 400 nm in a Bausch and Lomb Spectronic 200 UV spectrophotometer. Substrate was 1 mM *p*-nitrophenyl acetate in 0.1 M sodium phosphate at pH 6.5. Since substrate hydrolyzes on storage in aqueous solution, stock substrates were prepared in 0.1 M concentration in acetone and diluted 1:100 immediately before use. Substrate prepared in this manner was stable for 1 h. One unit of activity was arbitrarily defined as an increase of 1.0 absorbance unit at 400 nm per min in a 1 ml reaction volume with a 1 cm light path.

Assay for Proteolytic Activity

Assays for general proteolytic activity were performed with 12 mg of Azocoll (Calbiochem's brand of collagen with a dye covalently attached) in either 0.2 M sodium acetate buffer, pH 5.5, 0.2 M sodium phosphate buffer, pH 6.5, 0.2 M sodium phosphate buffer, pH 7.5, or 0.2 M ethyl morpholine acetate buffer, pH 8.6. Reaction mixtures were incubated for 1 to 2 h at 37°C. Prior to reading the amount of dye solubilized, 2 ml of glass distilled water were added to the reaction mixtures and the suspension filtered through glass wool. The amount of dye released was monitored at 520 nm in a 1 cm light path.

Protein Assays

Protein was determined by absorbance at 280 nm. An extinction of 1.0 was assumed for a solution of 1 mg/ml protein.

RESULTS AND DISCUSSION

The esterase activity of rabbit testis was purified from a 20 mM sodium phosphate, pH 6.5, buffer extract of acetone powder of rabbit testis as outlined in Table 1 (Bradford et al., 1976). All purifications were performed in 20 mM sodium phosphate buffer, pH 6.5. The 108-fold purified esterase preparation was used for all of the data reported here unless otherwise noted. No detectable proteinase activity was observed at any pH in the esterase preparations under the conditions described in Methods.

Since elastase is a proteinase that hydrolyzes nitrophenyl acetate but does not digest azocollagen (Mallory and Travis, 1975) it was necessary to eliminate any possibility of the esterolytic activity being due to elastase activity. N-acetyl-alanyl-alanyl-prolyl-alanyl-chloromethyl ketone, a specific elastase inhibitor, was assayed for its effect on elastase and on

TABLE 1.

Purification step	Specific activity mU/OD 280 nm	% Recovery	Fold purification
Crude extract	177	100	1.0
G-150 Sephadex	914	85	4.4
DEAE-cellulose	2,000	48	11.3
Con A Sepharose	19,200	14	108.5

Downloaded from https://academic.oup.com/biolreprod/article/15/1/102/2841675 by guest on 21 August 2022

TABLE 2.

Material tested	Esterase mU	Percent corona cells dispersed		
		0 hours	1 hour	2 hours
Rabbit testicular esterase	400	0	50	100
Tomato pectin methyl esterase	400	0	50	100
Control	0	0	0	0

esterase. The inhibition of elastase was performed according to the method of Mallory and Travis (1975) with 0.5 mM *tert*-butyl-oxycarbonyl-L-alanine-*p*-nitrophenyl ester as substrate and 100 μg/ml of the inhibitor. Elastase esterase activity was completely inhibited. Testicular esterase assayed at the same level of esterase activity and inhibitor maintained 96 percent of the original activity.

Esterase was observed to remove the corona radiata from cumulus-free rabbit ova. To eliminate the possibility of co-purifying a trace contaminant in the esterase preparation, a plant esterase was purified from tomatoes according to Pithawala et al. (1948). When equivalent number of spectrophotometric units of the highly purified tomato pectin methyl esterase and rabbit testicular esterase were incubated with cumulus-free rabbit ova, the corona cells were dispersed at the same rate (Table 2).

These observations indicate that an ester linkage is involved in the attachment of the cells of the corona radiata to each other and to the zona pellucida of the rabbit ovum. Since ester linkages are labile to alkali, the alkali-lability of the corona radiata was investigated. Complete dispersal of the corona radiata occurred in 2 h in several buffers at pH 8.0. On incubation at higher pH levels, the corona was dispersed more rapidly. On incubation at pH 7.5 or below, no dispersion effect was observed (Fig. 1).

These results are in direct conflict with those of Stambaugh et al. (1969). Repeated experiments in Tris buffer (Fig. 1) as well as phosphate, EDTA, and glycinate gave identical results for alkaline removal of corona cells. The observation of Stambaugh et al. (1969) that bicarbonate in Eagle's Medium sometimes dispersed the corona radiata of rabbit ova must in fact have been due to pH effects since the concentration of bicarbonate ions would always be high between pH 6.37 and 10.25 (carbonic acid, $pK_1 = 6.37$ and $pK_2 = 10.25$) with a maximum at pH 8.3. Addition of bicarbonate to any unbuffered medium would cause the pH to rapidly approach this pH level, a level that removes corona cells in 2 h. Variable results obtained by these authors undoubtedly arose from the formation of hydroxyl ions which invariably form in aged bicarbonate solutions in the absence of a CO_2 atmosphere.

Two definitive experiments show that the bicarbonate effects must have arisen incidentally. Ova were subjected to 250 mM bicarbonate, over four times the reported effective level, in the presence of a strongly buffered solution (0.2 M Tris HCl) to maintain the pH at 7.5 in the corona dispersion assay. Even after 24 h no corona cell dispersion was observed. To check the hypothesis that the pH of the bicarbonate containing solution was increased by incubation in the absence of CO_2 atmosphere, a solution of 100 mM sodium bicarbonate was incubated at 37°C in the absence of CO_2 to demonstrate the production of hydroxyl ions with time. The

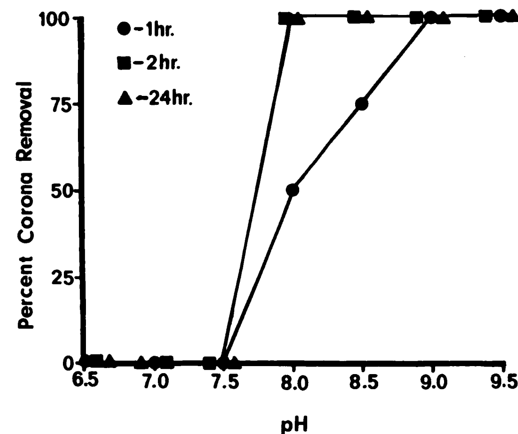

FIG. 1. pH Effects on the Corona Radiata of Rabbit Ova. 100 mM buffers at the pH's indicated were incubated with 4 cumulus-free rabbit ova in 0.4 ml volume at 37°C. Tris, phosphate, EDTA and glycinate buffers were used with virtually identical results. ● Status of ova at 1 h; ■ Status of ova at 2 h; ▲ Status of ova at 24 h.

Downloaded from https://academic.oup.com/biolreprod/article/15/1/102/2841675 by guest on 21 August 2022

pH rose from 8.30 to 8.59 in 1 h. The results of these studies show that bicarbonate ion has no effect on the corona radiata and that in actuality, the pH of a bicarbonate solution increases during incubation in the absence of CO_2 atmosphere. The active component in the observations of Stambaugh et al. (1969) must have been the pH effects that we have seen on the corona radiata of the rabbit ovum.

We have observed that the corona radiata of the rabbit ovum is dispersed by the action of the female reproductive tract in 6 to 8 h after ovulation confirming the report of Stambaugh et al. (1969). In our examination of biological samples for esterase activity, we determined that there was esterase present in rabbit acrosomal extracts, testicular extracts, seminal plasma, epididymal fluid and oviductal fluid. Partially purified esterase from seminal plasma, epididymal fluid and acrosomal extracts have also been demonstrated to remove the corona radiata from rabbit ova. The pH of the oviductal fluid of the rabbit is 7.8–8.2 (Hamner and Williams, 1965). Oviduct fluid alone through a combination of pH and esterolytic activity combined with the peristaltic activity of the oviduct should readily disperse the corona cells in the six to eight hour time period.

In preliminary experiments using rabbit sperm esterases on the large corona radiata of the cat ovum, the cat corona radiata is rapidly dispersed. Evidently, the esterase activity is not species specific.

Esterase activity in mammalian spermatozoa has been previously reported. Meizel et al. (1971) found many different esterases in head, midpiece, and tail extracts of bovine sperm. Bryan and Unnithan reported esterase activity in bovine acrosomes (1972) and in mouse acrosomes (1973) by cytochemical staining. Beckman and Jessler (1968) reported esterase isoenzymes in human spermatozoa. However, the present report is the first report of a role for esterases in the fertilization process.

The observation that esterases are involved in the dispersal, and perhaps the penetration, of the corona radiata of rabbit ova during fertilization, permits a classification of the cells comprising the follicular mass of the ovum on a biochemical basis. All follicular cells that disperse by the action of hyaluronidase may be considered to be cumulus cells. Oikawa et al. (1975) use hyaluronidase to disperse all of the follicular cells from hamster, mouse, and rat ova. In our laboratory all of the follicular cells of bovine ova are dispersed by 1 mg/ml hyaluronidase (Worthington, 473 N.F. units/mg) in Krebs Ringer Phosphate, pH 7.2, in approximately 15 min. On a biochemical basis, therefore, hamster, mouse, rat, and bovine ova possess only cumulus cells. All follicular cells that are not dispersable by hyaluronidase but are dispersable by high pH and esterase activity are best described as corona radiata cells. On a biochemical basis, rabbit and cat possess both cumulus and corona radiata cells in their follicular masses.

ACKNOWLEDGMENTS

This research was supported by Contract No. 1-HD-9-2103 with the Center for Population Research, NICHD and by Ford Foundation grant no. 680-0805A.

REFERENCES

Austin, C. R. (1948). Function of hyaluronidase in fertilization. Nature 162, 63-64.

Beckmann, L. and Jessler, B. K. (1968). Isoenzymes of human sperm esterase. Acta Genet. (Basel) 18, 55-59.

Bradford, M. M. and Lehnhardt, W. F. (1974). Purification of corona penetrating enzyme and inhibition by decapitation factor preparations. Abstracts, Seventh Annual Meeting, Society for the Study of Reproduction, August 19–22, Carleton University, Ottawa, Ontario, Canada.

Bradford, M. M. (1974). Biochemistry of ovum penetration. Master's Thesis, University of Georgia.

Bradford, M. M. (1975). Acrosomal proteinases other than acrosin. Abstracts Eighth Annual Meeting, Society for the Study of Reproduction, July 22–25, Colorado State University, Fort Collins, Colorado.

Bradford, M. M., McRorie, R. A. and Williams, W. L. (1976). Biochemical characterization and zymographic measurement of the non-specific esterase of rabbit testis. In manuscript.

Brantner, J. H., Medicus, R. G., Srivastava, P. N., Williams, W. L. and McRorie, R. A. (1974). Purification of proteinases in fertilization by affinity chromatography. Fed. Proc. 33, 1378.

Bryan, J. H. D. and Unnithan, R. R. (1972). Non-specific esterase activity in bovine acrosomes. Histochem. J. 4, 413-419.

Bryan, J. H. D. and Unnithan, R. R. (1973). Cytochemical localization of non-specific esterase and acid phosphatase in spermatozoa of the mouse *(Mus musculus)*. Histochemie 33, 169.

Chang, M. C. (1950). Fertilization, male infertility and hyaluronidase. N. Y. Acad. Sci. 52, 1192-1195.

Hamner, C. E. and Williams, W. L. (1965). Composition of rabbit oviductal secretions. Fertil. Steril. 16, 271-282.

Mallory, P. A. and Travis, J. (1975). Human pancreatic enzymes: Purification and characterization of a nonelastolytic enzyme, protease E, resembling elastase. Biochem. 14, 722-730.

Meizel, S., Boggs, D. and Cotham, J. (1971). Electro-

Downloaded from https://academic.oup.com/biolreprod/article/15/1/102/2841675 by guest on 21 August 2022

phoretic studies of esterases of bull spermatozoa, cytoplasmic droplets and seminal plasma. J. Histochem. Cytochem. 19, 226-231.

Oikawa, J., Yanagimachi, R. and Nicolson, G. L. (1975). Species differences in the lectin-binding sites on the zona pellucida of rodent eggs. J. Reprod. Fert. 43, 137-140.

Pincus, G. and Enzmann, E. V. (1932). Fertilization in the rabbit. J. Exp. Zool. 9, 403-408.

Pincus, G. and Enzmann, E. V. (1936). The comparative behavior of mammalian eggs *in vivo* and *in vitro*. II. The activation of tubal eggs of the rabbit. J. Exp. Zool. 13, 195-208.

Pithawala, H. R., Savur, G. R. and Sreenivasan, A. (1948). Characterization of tomato pectinesterase. Arch. Biochem. 17, 235-248.

Srivastava, P. N., Adams, C. E. and Hartree, E. F. (1965a). Enzymic action of a lipoglycoprotein preparation from sperm acrosomes on rabbit ova. Nature 205, 498.

Srivastava, P. N., Adams, C. E. and Hartree, E. F. (1965b). Enzymatic action of acrosomal preparations on the rabbit ova *in vitro*. J. Reprod. Fert. 10, 61-67.

Stambaugh, R., Noriega, C. and Mastroianni, L. (1969). Bicarbonate ion: The corona dispersing factor of rabbit tubal fluid. J. Reprod. Fert. 18, 51-58.

Tillman, W. L. (1972). Enzymology of spermatozoan penetration of mammalian ova. Master's Thesis, University of Georgia.

Yamane, J. (1935a). Kausel-analytischen Studien über die Befruchtung des Kanincheneies. I. Die Dispersion der Follikelzellen und die Absolung der Zellen der Corona Radiata des Eies durch Spermatozoen. Cytologia 6, 233-255.

Yamane, J. (1935b). Kausel-analytischen Studien über die Befruchtung des Kanincheneies. II. Die Isolierung der auf das Eizytoplasma auflusund wirkenden Substanzen aus den Spermatozoen. Cytologia 6, 475-483.

Zaneveld, L. J. D., McRorie, R. A. and Williams, W. L. (1969). Enzymatic removal of the corona radiata of the ovum. Fed. Proc. 28, 705.

Zaneveld, L. J. D. and Williams, W. L. (1970). A sperm enzyme that disperses the corona radiata and its inhibition by decapacitation factor. Biol. Reprod. 2, 363-368.

Downloaded from https://academic.oup.com/biolreprod/article/15/1/102/2841675 by guest on 21 August 2022