Limited proteolysis of the porcine zona pellucida by homologous sperm acrosin

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Summary. Boar sperm acrosin isolated by affinity chromatography on *p*-(*p*'-aminophenoxypropoxy)benzamidine linked to Sepharose was tested for its proteolytic effect on the zona pellucida of freshly ovulated pig eggs. During 1 h in a physiological medium there was no observable change in the morphology of the zona pellucida but subsequent ¹²⁵I labelling of the structure followed by electrophoretic analysis revealed that acrosin had asserted a limited and selective proteolysis.

Introduction

For the mammalian spermatozoon to fertilize it must penetrate the zona pellucida, a relatively thick species-specific glycoprotein layer enveloping the egg (Dunbar, 1983). It is thought that the spermatozoon achieves this through exposure of hydrolytic enzymes associated with its acrosome which then assert a direct lytic action on the investment. A major, if not total, role in this penetration process has been attributed to the serine proteinase acrosin (EC 3.4.21.10: for references see Brown, 1982) although this assignation has been questioned (Morton, 1977; Bedford & Cross, 1978; Brown, 1982; Huneau, Harrison & Fléchon, 1984). One reason for doubt is that there is evidence for other roles for acrosin in fertilization (Meizel & Lui, 1976; Green, 1978; Saling, 1981) and another is that in some species, such as sheep and pig, an effect of pure acrosin on the gross morphology of the zona pellucida has yet to be demonstrated (Brown, 1982). However, it remains possible that the enzyme's action on the zona pellucida is a limited one, detectable only at the molecular level, and if this were so it would strengthen the concept that several acrosomal hydrolases including acrosin act synergistically on the zona pellucida to facilitate sperm penetration (McRorie & Williams, 1974; Brown, 1982, 1983). Studies on the structure of the porcine zona pellucida have utilized preparations collected on a large scale from ovaries (Gwatkin, Anderson & Williams, 1980; Dunbar, Wardrip & Hedrick, 1980) and have established that it consists of three major glycoproteins (Dunbar, 1983) the largest possessing a molecular weight of ~95 000. In the present attempt to detect an effect of boar acrosin on the zona pellucida at the molecular level we used individual zonae pellucidae still attached to eggs freshly recovered from the oviduct.

Materials and Methods

Chemicals. All chemicals were of the highest purity available and obtained from sources previously described (Brown, Andani & Hartree, 1975; Brown & Hartree, 1978). ¹⁴C-Methylated molecular weight marker proteins and the reagent *N*-succinimidyl 3-(4-hydroxy,5-[¹²⁵I]iodophenyl) propionate were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. PMSG and hCG were purchased from Intervet Laboratories Ltd, Cambridge, U.K.

Collection of eggs. Cross-bred (Large White and Landrace) gilts (aged 8–10 months) were injected with 1250 i.u. PMSG on Day 16 of the oestrous cycle followed by 500 i.u. hCG on Day 19.5. At 42–44 h after hCG the animals were anaesthetized and eggs were flushed from the oviducts in Dulbecco's phosphate-buffered saline, pH 7.4 (Dulbecco & Vogt, 1954) supplemented with 0.33 mM-sodium pyruvate, 6 mM-glucose, 4 mg bovine serum albumin (BSA)/ml and 0.025 mg kanamycin sulphate/ml (medium). Eggs were either recovered devoid of corona radiata cells, i.e. with the zona pellucida outermost, or such cells were removed by gentle suction of the eggs into and out of a fine-bored Pasteur pipette. Before use eggs were washed thoroughly in medium without BSA, and for subsequent manipulation of eggs and zonae pellucidae BSA was omitted.

Spermatozoa. Spermatozoa from the sperm-rich fraction of cross-bred boar ejaculates were washed in sucrose solutions to remove seminal plasma and cytoplasmic droplets (Brown *et al.*, 1975) and suspended in 0.25 M-sucrose for fractionation procedures.

Determination of acrosin activity. Acrosin activity was measured spectrophotometrically using *N*- α -benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) as substrate (Brown *et al.*, 1975) and is expressed as international units (μ mol substrate hydrolysed/min).

Purification of boar acrosin. This was achieved essentially according to the procedure previously described for the purification of ram β -acrosin (Brown & Hartree, 1978). The pre-chromatography protocol used to obtain fraction S6 from ram spermatozoa was followed and, briefly, entailed removal of an acrosin inhibitor from spermatozoa by 'denudation' of washed cells, followed by preparation of a crude extract from which the enzyme was purified using an affinity column containing Sepharose 4B linked to the acrosin inhibitor *p*-(*p*'-aminophenoxypropoxy)benzamidine. However, column chromatography conditions were modified from those previously described in that, after sample application and thorough washing of the column with 0.4 M-NaCl, 50 mM-Tris, 20 mM-CaCl₂ pH 8, elution of acrosin was carried out with 0.3 M-NaCl, 10 mM-citrate pH 2.5. Previously described (Brown & Hartree, 1978) post-column procedures were employed to yield a stable freeze-dried preparation which gave a single band at a molecular weight position of 34 000 on sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis run under reducing conditions. The enzyme possessed a specific activity of 230 i.u./mg when freshly dissolved and assayed under optimum conditions (Brown *et al.*, 1975). However, its activity towards Bz-Arg-Oet was reduced by 60% in the medium used for egg-acrosin incubations.

Digestion of zonae pellucidae with acrosin. Groups of ~10 eggs with the zona pellucida outermost were incubated at 39°C in acrosin dissolved at 0.5 mg/ml medium (39°C represents the core temperature in the domesticated species and 7.4 the pH of the oviducal fluid at oestrus). After 5, 15, 30 and 60 min they were transferred, in about 0.05 ml, into 2 ml 0.02 M-*p*-aminobenzamidine in medium to arrest proteolysis, followed by three washes in medium alone. Controls consisted of eggs similarly incubated in medium with and without 0.02 M-*p*-aminobenzamidine and of acrosin incubated at 0.5 mg/ml in medium for the same periods, after which 20 μ l samples were added to 2 μ l 0.2 M-*p*-aminobenzamidine. During the incubations the morphology of the zonae pellucidae was assessed microscopically at $\times 80$ magnification. The activity of acrosin was tested at the beginning and end of the incubation using Bz-Arg-OEt as substrate. For ¹²⁵I labelling each zona pellucida was carefully removed from the egg with a Leitz micro-manipulator and suspended in 10 μ l medium.

¹²⁵I-labelling of zonae pellucidae and acrosin. To ~10 zonae pellucidae in 10 μ l medium was added 2.5 μ Ci reagent (Bolton & Hunter, 1973) in 20 μ l 0.1 M-borate buffer pH 8.5. After 15 min at 25°C the zonae were washed three times in medium and dissolved individually in sample buffer for electrophoresis (see below). Labelling of acrosin controls was carried out using the same concentration of reagent as for zonae pellucidae, after which protein was precipitated with 9 volumes of cold ethanol and dissolved in electrophoresis sample buffer.

Fig. 1. Time-course of digestion of pig zona pellucida with boar acrosin analysed on non-reducing SDS/polyacrylamide gel electrophoresis. **Track a**, zona pellucida from an egg incubated in medium + *p*-aminobenzamidine (control); **tracks b, d, f and h**, acrosin incubated in medium for 5, 15, 30 and 60 min respectively before labelling (controls); **tracks c, e, g and i**, zonae pellucidae from eggs incubated in medium + 0.5 mg acrosin/ml for 5, 15, 30 and 60 min respectively. Tracks a, c, e, g and i received 0.25 of a zona pellucida ($\sim 100\,000$ c.p.m.).

Electrophoresis. Labelled zonae pellucidae and ethanol precipitated acrosin were dissolved in sample buffer consisting of 62.5 mM-Tris-HCl pH 6.8, 2% (w/v) SDS and 10% (w/v) glycerol and analysed on non-reducing 5–20% (w/v) polyacrylamide gradient slab gels (80 mm × 100 mm × 0.5 mm) containing 0.1% SDS. After electrophoresis ^{125}I -labelled proteins were detected by autoradiography on Kodak X-Omat S film.

Results

^{125}I labelling of the zona pellucida of eggs recovered from the oviduct followed by non-reducing SDS/polyacrylamide gel electrophoresis consistently revealed major polypeptides of M_r about 250 000, 90 000 and 79 000 with a broader area of label spread between M_r 68 000 and 50 000. This pattern was unaltered by incubation of zonae at 39°C for 1 h in Dulbecco's medium with or without 20 mM-*p*-aminobenzamidine (Pl. 1, Fig. 1, track a). However, after only 5 min exposure to acrosin the M_r 250 000 polypeptide had disappeared and the M_r 90 000 polypeptide virtually so, giving rise to the appearance of polypeptides at M_r about 210 000, 150 000, 114 000, 65 000 and 57 000 and a doublet at $M_r \sim 50$ 000 (Pl. 1, Fig. 1, track c). The polypeptides of M_r 210 000, 150 000 and 114 000 appeared to be hydrolysis products of the polypeptide of M_r 250 000 while those of M_r 65 000 and 57 000 and the doublet components could be from any higher molecular weight precursor. After exposure to acrosin for 15 min (Pl. 1, Fig. 1, track e) the polypeptides of M_r 210 000, 150 000 and 90 000 had disappeared. During the next 45 min of incubation with acrosin there was essentially no further change of the electrophoretic profile although the level of acrosin activity had fallen by 40%. The egg and the zona pellucida were morphologically intact as judged by light microscopy.

Discussion

Important features of these experiments were (i) the use of fully matured eggs from the oviduct, (ii) the incubation of enzyme with the intact zona pellucida *in situ* on viable eggs, (iii) the use of a method for removing the zona pellucida from individual eggs which causes no fragmentation of the investment and (iv) the electrophoretic analysis of a single zona pellucida. Although zonae pellucidae looked morphologically intact after incubation with acrosin it is clear from Pl. 1, Fig. 1 that they had undergone a limited and selected proteolysis. The polypeptide of M_r 90 000 from the zona pellucida was quickly hydrolysed while that of M_r 79 000 appeared to be acrosin-resistant in the incubation conditions employed. The fate, if any, of the material at M_r 50–68 000 cannot be assessed from Pl. 1, Fig. 1. Hydrolysis of the protein of M_r 250 000, which the zona pellucida apparently absorbs from the oviduct, was also rapid and produced an acrosin-resistant polypeptide of M_r 114 000 which, like that of M_r 250 000, remained bound to the zona pellucida in spite of several washes. *In vivo*, the boar spermatozoon, once having begun penetration of the zona pellucida traverses it in a matter of minutes rather than hours, so evidently any hydrolysis of zona pellucida moieties is fairly rapid. In the present experiments proteolysis by acrosin, although limited, was indeed rapid. Even so the concentration of enzyme chosen for the tests (0.5 mg/ml) could be far less than the spermatozoon carries to the zona pellucida, because the concentration of this particular enzyme in the acrosome of the ram spermatozoon is ~ 40 mg/ml (Brown & Hartree, 1976). The molecular species of boar acrosin isolated for the present tests by the acrosin inhibitor affinity adsorbent appears to be m β -acrosin as judged by its molecular weight of 34 000 (Parrish & Polakoski, 1978). This molecular weight was obtained from SDS/polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, but under non-reducing conditions the enzyme appears to migrate with an apparent M_r of 38 000 (Pl. 1, Fig. 1).

The observed limited proteolysis of the pig zona pellucida by homologous acrosin strengthens the case for a role for the enzyme in sperm penetration through this investment and also provides a

contrast to the mouse in which homologous acrosin will digest and completely remove the zona pellucida from the egg (Brown, 1983). This suggests that in the mouse the action of acrosin alone may facilitate sperm penetration. In the pig, however, acrosin's limited proteolysis of the zona pellucida might modify the investment sufficiently to permit a solely acrosin-mediated sperm penetration (non-enzymic factors aside), or could be an indication that the spermatozoon does indeed utilize a synergism of acrosomal hydrolases for this purpose.

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References

- Bedford, J.M. & Cross, N.L. (1978) Normal penetration of rabbit spermatozoa through trypsin- and acrosin-resistant zona pellucida. *J. Reprod. Fert.* **54**, 385–392.
- Bolton, A.E. & Hunter, W.M. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent. *Biochem. J.* **133**, 529–539.
- Brown, C.R. (1982) Effects of ram sperm acrosin on the investments of sheep, pig, mouse and gerbil eggs. *J. Reprod. Fert.* **64**, 457–462.
- Brown, C.R. (1983) Purification of mouse sperm acrosin, its activation from proacrosin and effect on homologous egg investments. *J. Reprod. Fert.* **69**, 289–295.
- Brown, C.R. & Hartree, E.F. (1976) Effect of acrosin inhibitors on the soluble and membrane-bound forms of ram acrosin, and a reappraisal of the role of the enzyme in fertilization. *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 57–65.
- Brown, C.R. & Hartree, E.F. (1978) Studies on ram acrosin. Activation of proacrosin accompanying the isolation of acrosin from spermatozoa and purification of the enzyme by affinity chromatography. *Biochem. J.* **175**, 227–238.
- Brown, C.R., Andani, Z. & Hartree, E.F. (1975) Studies on ram 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.* **99**, 167–199.
- Dunbar, B. S. (1983) Morphological, biochemical and immunochemical characterization of the mammalian zona pellucida. In *Mechanism and Control of Animal Fertilization*, pp. 139–175. Ed. J. F. Hartmann. Academic Press, New York.
- Dunbar, B.S., Wardrip, N.J. & Hedrick, J.L. (1980) Isolation, physicochemical properties, and macromolecular composition of zona pellucida from porcine oocytes. *Biochemistry, N.Y.* **19**, 356–364.
- Green, D.P.L. (1978) The activation of proteolysis in the acrosome reaction of guinea-pig sperm. *J. Cell Sci.* **32**, 153–164.
- Gwatkin, R.B.L., Anderson, O.F. & Williams, D.T. (1980) Large scale isolation of bovine and pig zonae pellucidae: chemical, immunological and receptor properties. *Gamete Res.* **3**, 217–231.
- Huneau, D., Harrison, R.A.P. & Fléchon, J.E. (1984) Ultrastructural localization of proacrosin and acrosin in ram spermatozoa. *Gamete Res.* **9**, 425–440.
- McRorie, R.A. & Williams, W.L. (1974) Biochemistry of mammalian fertilization. *A. Rev. Biochem.* **43**, 777–803.
- Meizel, S. & Lui, C.W. (1976) Evidence for the role of a trypsin-like enzyme in the hamster sperm acrosome reaction. *J. exp. Zool.* **195**, 137–144.
- Morton, D.B. (1977) The occurrence and function of proteolytic enzymes in the reproductive tract of mammals. In *Proteinases in Cells and Tissues*, pp. 445–500. Ed. A. J. Barrett. North Holland, Amsterdam.
- Parrish, R.F. & Polakoski, K.L. (1978) Boar α -acrosin. Purification and characterization of the initial active enzyme resulting from the conversion of boar proacrosin to acrosin. *J. biol. Chem.* **253**, 8428–8432.
- Saling, P.M. (1981) Involvement of trypsin-like activity in binding of mouse spermatozoa to zonae pellucidae. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6231–6235.

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