Purification of mouse sperm acrosin, its activation from proacrosin and effect on homologous egg investments

C. R. Brown

A.R.C. Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road,
Cambridge CB3 0JQ, U.K.

Summary. When proacrosin from mouse epididymal spermatozoa was activated a single form of acrosin was produced. The enzyme was isolated by gel filtration followed by affinity chromatography using Sepharose-4B linked to an acrosin inhibitor *p*-(*p*'-aminophenoxypropoxy)benzamidine. The molecular weight of partly purified acrosin was 53 000 by gel filtration, and of the pure enzyme 39 000 by SDS-polyacrylamide gel electrophoresis. Pure mouse acrosin removed the cumulus oophorus, corona radiata and zona pellucida from the homologous egg. It is proposed that penetration of spermatozoa through egg investments, particularly through the zona pellucida, is a simpler process in the mouse than in the sheep.

Introduction

The contention that the digestive action of the acrosomal proteinase, acrosin (EC 3.4.21.10), on the egg zona pellucida is the means by which the spermatozoon is able to penetrate this investment during fertilization has recently been subject to scrutiny (Bedford & Cross, 1978). The main evidence in support of the proposition is that inhibitors of proteinase activity impair fertilization *in vivo* and *in vitro* (Stambaugh & Buckley, 1969; Stambaugh, Brackett & Mastroianni, 1969; Zaneveld, Robertson, Kessler & Williams, 1971; Yang, Zaneveld & Schumacher, 1976; Beyler & Zaneveld, 1982; Fraser, 1982), and that preparations of acrosin will, under certain experimental conditions, remove the zona pellucida from the ovum (Stambaugh, & Buckley, 1969; Meizel & Mukerji, 1976; Brown, 1982). However, the inhibitors used were not specific to acrosin and tests on the ability of acrosin to disperse the zona pellucida have often been carried out with impure enzyme in a non-physiological medium or with acrosin and egg from different species. Furthermore, there is evidence of a role for acrosin either in the acrosome reaction itself (Lui & Meizel, 1979) or in dispersal of the acrosomal matrix once this reaction has occurred (Green, 1978).

It has been demonstrated that under conditions *in vitro*, akin to physiological, pure ram β -acrosin will not remove the zona pellucida from the sheep egg whereas it will rapidly remove all investments from the mouse egg (Brown, 1982). This observation suggests that, in the sheep, sperm penetration of the zona pellucida is aided by enzymes other than, or in addition to, acrosin. It also suggests that the mouse zona pellucida might be demonstrably susceptible to pure mouse acrosin. Since this has not previously been tested the present paper describes the complete purification of mouse acrosin and its effect on the investments of homologous eggs. Because mouse acrosin is unstable and present in spermatozoa in small amounts it was necessary to accrue the enzyme in its stable zymogen form, proacrosin, before purification studies. This was achieved by collecting and storing spermatozoa in a medium containing *p*-aminobenzamidine, an inhibitor of acrosin which also reversibly blocks the activation of proacrosin to acrosin (Brown & Harrison, 1978). Consequently, new data on the activation of mouse proacrosin are also presented.

Materials and Methods

Collection and preparation of epididymal spermatozoa

Epididymides were excised from freshly killed 3–4-month-old Carworth Europe CLFP non-inbred mice and the cauda epididymidis diced with a scalpel in 0.264 M-sucrose, 5 mM-MES, 0.5 mM-*p*AB (*p*-aminobenzamidine hydrochloride) pH 6.5 (sucrose-*p*AB buffer), at 20°C. Spermatozoa were collected from groups of 50 mice and diluted into 20 ml buffer, filtered through siliconized glass wool to remove contaminating tissue and centrifuged for 30 min at 3000 *g* (4°C). Spermatozoa were then resuspended in approximately 4 ml sucrose-*p*AB buffer and stored at –20°C until use. When needed, the spermatozoa were thawed, diluted in sucrose-*p*AB to approximately 2×10^8 cells/ml and denuded, i.e. the outer acrosomal membrane and acrosomal contents were released by vortex mixing (Brown, Andani & Hartree, 1975). This procedure, which also releases endogenous acrosin inhibitor(s) (Hartree, 1977), results in little or no proacrosin being lost from the spermatozoa. Denuded spermatozoa were then washed free of *p*AB and detached cell components by centrifuging (25 min at 1500 *g*) 2 ml aliquants through 8 ml steps of 0.3 M-sucrose, 5 mM-MES, pH 6.0 and resuspended in appropriate medium for each experiment.

Collection of eggs

Eggs were collected into a balanced medium (Brown, 1982) from the oviducts of 26–28-day-old CLFP mice treated previously with 12 i.u. PMSG (48 h) and 12 i.u. hCG (18 h), and used without delay. The eggs were left in the cumulus, denuded of all but the corona radiata, or freed entirely of follicle cells by gentle suction into and out of a fine-bored Pasteur pipette to leave the zona pellucida outermost. Incubations with enzyme were carried out on siliconized watch glasses.

Incubation of eggs with acrosin

Preliminary experiments showed purified mouse acrosin to be very unstable at physiological pH; thus the enzyme, when adjusted to pH 7.4 with Hepes, lost approximately 50% of its activity after 5 min at 37°C and 90% after 40 min. Because of this instability egg-acrosin incubations were carried out as follows. Eggs, with cumulus oophorus or corona radiata or the zona pellucida outermost, were collected into groups of 12–15 in approximately 10 µl medium (see 'Materials and Methods'). Acrosin, freshly purified, was kept at 4°C in 0.15 M-NaCl, 5 mM-CaCl₂ pH 2.6 and an aliquant (8 µl) was adjusted to pH 7.4 with 0.4 µl 0.2 M-Hepes pH 7.7. This was added without delay to the eggs in medium, the mixture covered with liquid paraffin at 37°C and incubated at 37°C for 40 min. The eggs were then transferred in approximately 10 µl medium to a freshly neutralized 8 µl aliquant of acrosin, covered in liquid paraffin, and the incubation at 37°C continued. This process was repeated at 40 min intervals during which observations on the egg investments were made. Control eggs, grouped according to which investment was outermost, received the same treatment less enzyme.

Acrosin assays

Fluorometric assays with benzoylarginine-2-naphthylamide (BANA) as substrate were carried out essentially as described by Brown & Hartree (1976). Assays were at 25°C in a system (0.5 ml) containing 50 mM-Tris-HCl, 0.2 M-CaCl₂, 10% v/v dimethylsulphoxide, 1 mM-BANA and sample, pH 8.2. The fluorometer was calibrated with 0–50 pmol 2-naphthylamine measured in the assay system (less enzyme only). Enzyme activity is expressed in mU, i.e. nmol BANA hydrolysed per min, unless stated otherwise.

Electrophoretic analyses

Electrophoresis on non-denaturing polyacrylamide gels (70 mm × 6 mm) at pH 4.9 and subsequent detection of acrosin and proacrosin in gels with benzoylarginine-2-naphthylamide and Fast Garnet GBC was carried out as detailed by Harrison (1982).

Electrophoresis on 12% polyacrylamide gels containing SDS was carried out under reducing and non-reducing conditions according to Jones, Brown, Von Glos & Parker (1980). Molecular weight standards were bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α -lactalbumin (14 400). Proteins were detected with a silver stain by the method of Poehling & Neuhoff (1981).

Gel filtration on Sephadex G100

A column (90 cm × 2 cm²) was calibrated with bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen (25 000) and ribonuclease (13 700). The void volume was determined with blue dextran, and the column buffer was 0.15 M-NaCl, 5 mM-CaCl₂ pH 2.6. Fractions of 2 ml were collected in plastic tubes at a flow-rate of 12 ml/h and all runs were at 4°C. The method of Andrews (1964) was used to estimate the molecular weight of acrosin.

Affinity chromatography

Acrosin, partly purified by chromatography on Sephadex-G100, was isolated using a column (6 cm × 1 cm²) of Sepharose-4B linked to an acrosin inhibitor *p*-(*p*'-aminophenoxypropoxy) benzamidine (Brown & Hartree, 1978). The design of subsequent egg-acrosin tests required that buffers used for binding and elution of acrosin were modified from those previously described. Therefore the column was equilibrated for binding with 0.15 M-NaCl, 5 mM-CaCl₂, 5 mM-Tris pH 8, and the enzyme was eluted with 0.15 M-NaCl, 5 mM-CaCl₂ pH 2.6. Application and elution of sample was at 4°C at a flow rate of 8 ml/h.

Results

Time-course and electrophoretic analysis of the activation of mouse epididymal sperm proacrosin

As a preliminary step in the purification of mouse sperm acrosin it was necessary to study the time-course of proacrosin activation and observe the active enzyme form(s) produced in a suspension of denuded spermatozoa (Text-fig. 1). Since there was no acrosin in the supernatant from which the denuded spermatozoa were centrifuged (as revealed by incubating the supernatant at pH 3 before enzyme assay) it appears that there is no active enzyme present in the intact spermatozoon. Activation proceeded rapidly after removal of *p*-aminobenzamidine to give maximum activity after 40–60 min. When the activation was followed on non-denaturing polyacrylamide gel electrophoresis, only a single molecular species of acrosin was seen, slightly more basic than proacrosin (Text-fig. 1). The active species of acrosin was detectable after 10-min incubation at a time when total enzyme activity had reached only 10% of the maximum as measured by BANA hydrolysis. Throughout the entire activation process the charge on the active species remained unaltered.

Purification of acrosin from mouse epididymal spermatozoa

Denuded spermatozoa (4.5×10^9) from 400 mice were washed free of *p*AB (see 'Materials and Methods') and resuspended in 28 ml 0.264 M-sucrose, 2 mM-Hepes pH 7.5. They were incubated at 25°C until maximum acrosin activity was reached (Text-fig. 1), made 0.15 M in NaCl, 5 mM in

Text-fig. 1. Time-course (a) and electrophoretic analysis (b) of the activation of mouse epididymal proacrosin. Epididymal spermatozoa from 75 mice were denuded, washed free of pAB (see 'Materials and Methods') and resuspended at a concentration of 8×10^7 cells/ml in 6 ml 0.264 M-sucrose, 2 mM-Hepes pH 7.5 at 25 °C. At timed intervals 1.0 ml aliquants were centrifuged (4°C, 10 000 g) for 5 min and pelleted spermatozoa were resuspended in 0.15 ml 0.25 M-sucrose-HCl pH 2.6. After 30 min at 4°C the suspensions were centrifuged at 10 000 g and the supernatants containing the proacrosin-acrosin were assayed for acrosin activity and analysed on non-denaturing polyacrylamide gels at pH 4.9. Each gel received 0.1 ml proacrosin-acrosin preparation containing 0.6 mU potential activity.

CaCl₂ and then acidified to pH 2.6 with 1 N-hydrochloric acid. After 40 min at 4°C they were centrifuged for 40 min at 30 000 g (4°C) and the supernatant concentrated to 3 ml in an Amicon ultrafiltration cell fitted with a PM10 membrane. This preparation, containing 38 mU acrosin activity, was passed through the Sephadex G100 column to ensure complete removal of inhibitors. It emerged as a single peak with 24 mU activity and with a molecular weight of 53 000. Acrosin-containing fractions were pooled and concentrated in the ultrafiltration cell to 3 ml, adjusted to 5 mM in CaCl₂ and then to pH 8 with careful addition of 1 M-Tris. Without delay it was applied to the Sepharose 4B-*p*-(*p*'-aminophenoxypropoxy) benzamidine column, which was then washed thoroughly with 0.15 M-NaCl, 5 mM-CaCl₂, 5 mM-Tris pH 8. The total wash through contained only a trace of acrosin activity. Bound acrosin was then eluted with 0.15 M-NaCl, 5 mM-CaCl₂ pH 2.6 and fractions containing activity were combined, adjusted to pH 2.6 with HCl and concentrated to 200 µl in an Amicon ultrafiltration cell fitted with a PM10 membrane.

This preparation contained 12 mU acrosin activity and gave a protein band with a molecular weight of 39 000 when electrophoresed under reducing and non-reducing conditions on SDS-polyacrylamide gels followed by silver staining (Text-fig. 2).

Effect of pure mouse acrosin on the investments of mouse eggs

In the presence of enzyme the cumulus oophorus began to disperse within a few minutes and by 15 min both it and the corona radiata were completely removed. After this time investments on the relevant control eggs were retained. By 1.5 h erosion of the outer surface of the zona pellucida was evident and by 6 h the investment was completely removed. The zona pellucida of control eggs by this time was visibly unaffected. Effects of pure acrosin were very consistent from egg to egg. Inspection of control eggs after a further 14 h showed the zona pellucida and corona radiata to be present and of normal appearance, whilst the cumulus oophorus had dispersed.

To confirm that dispersal of the cumulus oophorus and corona radiata was acrosin-mediated the effect of the acrosin inhibitor *p*-aminobenzamidine was tested. At 5 mM concentration it prevented

Text-fig. 2. SDS-polyacrylamide gel electrophoretogram of mouse acrosin purified by gel filtration on Sephadex G-100 and affinity chromatography on Sepharose 4B-*p*-(*p*'-aminophenoxypropoxy) benzamidine.

dispersal of both investments (in tests on the corona, the cumulus had first been removed mechanically); after 4 h (longer times not tested) in the presence of enzyme and inhibitor the investments remained intact, whereas in enzyme alone both were removed within 15 min.

Discussion

Detailed data on the conversion of proacrosin to acrosin in rodent species, particularly the mouse, are lacking. Bhattacharyya, Goodpasture & Zaneveld (1979) detected significant amounts of acrosin in mouse epididymal spermatozoa but the present results indicate that virtually all the enzyme is present in the zymogen form because no active acrosin could be detected in spermatozoa freed of *p*-aminobenzamidine or in fractions derived from denuded spermatozoa. Thus, mouse spermatozoa appear to be similar to those of several other species such as ram, bull and boar (Brown & Harrison, 1978). The time taken for mouse sperm proacrosin to fully activate (40 min) is also similar to that seen in other species, but the transformation of the zymogen to the enzyme appears to be unusual in that it is a single-stage process which occurs before the enzyme acquires full catalytic activity (Text-fig. 1). This delay was not due to interference by acrosin inhibitor slowing down the transformation. If this was the case, proacrosin as well as acrosin would have been visualized in gels of partly activated samples, for instance after 10 min activation (Text-fig. 1). It is more likely that this delay in development of full catalytic activity reflects a change in the configuration of the acrosin molecule which involves no further change in charge. In other species

so far examined the activation of sperm proacrosin generates multiple forms of acrosin which vary in stability. Usually one form is sufficiently stable to predominate, making its isolation possible, as in the case of ram β -acrosin (Brown & Hartree, 1978). It would seem reasonable to suppose that these dominant forms of acrosin are those which function *in vivo*, but this remains to be proved; all forms could function together, although it is possible that a minor and/or transient form could play a key role, especially forms that are generated in the early stages of proacrosin activation. Therefore, a degree of uncertainty must exist as to the physiological relevance of one enzyme form which is selected from a number for tests in acrosin-egg incubations *in vitro*. In the mouse there is no such uncertainty because the activation of proacrosin produces only a single form of enzyme.

The inherent instability of mouse acrosin (Brown & Hartree, 1976) and its presence in spermatozoa in comparatively low amounts have together made its isolation difficult. Although the purification procedure was carried through without delay and the length of time that the enzyme was subjected to pH 8 for affinity chromatography kept to a minimum, there was an overall loss of 68% of enzyme. This loss was not, however, as dramatic as the inactivation of the pure enzyme in egg-incubation medium at 37°C. The value of 39 000 for the molecular weight of pure mouse acrosin as determined by SDS-polyacrylamide gel electrophoresis differed considerably from that of the partly purified preparation (mol. wt 53 000) repeatedly obtained by gel filtration at pH 2-6. There may be two possible explanations for this. Firstly, the higher value may reflect a high carbohydrate content of the molecule. For example, pure boar acrosin, which contains 10% carbohydrate, has an apparent molecular weight of 56 000 by gel filtration (Fink, Schiessler, Arnhold & Fritz, 1972) and one of 38 000 by SDS-polyacrylamide gel electrophoresis (Schleuning & Fritz, 1974). Ram β -acrosin, with 3% carbohydrate, also shows a similar but smaller discrepancy and exhibits molecular weights of 45 000 and 38 000 by the respective methods (Brown & Hartree, 1978). Alternatively, the partly purified enzyme may, during gel filtration at pH 2-6, bind with a protein of lower molecular weight which is subsequently dissociated at pH 8 and separated from acrosin on the affinity column.

Inhibition of mouse *in-vitro* fertilization by synthetic acrosin inhibitors has been demonstrated. In one study (Beyler & Zaneveld, 1982) a range of inhibitors, which included *p*-aminobenzamidine, all prevented fertilization, the effectiveness of each inhibitor being commensurate with its ability to inhibit a partly purified mouse acrosin. This showed the action of a proteinase(s), probably acrosin, to be crucial in the fertilization process but cast no light on whether the enzyme(s) functions during the acrosome reaction and subsequent dispersal of the acrosomal matrix or during sperm penetration of egg investments. In another study to test the effect of *p*-aminobenzamidine on *in-vitro* fertilization of zona-free and zona-intact eggs, the results were consistent with a role for a *p*-aminobenzamidine-sensitive proteinase in penetration of the zona pellucida (Fraser, 1982). However, since the inhibitors used in both studies were not acrosin-specific the results were suggestive but not conclusive.

The present results show that in a physiological medium pure mouse acrosin will digest and completely remove the zona pellucida from homologous eggs as well as disperse the corona radiata and cumulus oophorus. This clearly indicates a role for mouse acrosin in digestion of the zona pellucida and also implies that hydrolases other than acrosin are unnecessary for sperm penetration in the mouse, although they may be needed for maximum efficiency of penetration. However, very little is known of the hydrolase content of the mouse sperm acrosome, that is, whether or not it sequesters a range of enzymes similar to those detected in other species (McRorie & Williams, 1974). Mouse acrosin dispersed the cumulus oophorus and corona radiata much more rapidly than it removed the zona pellucida, which was also the case when the effect of pure ram acrosin on mouse egg investments was tested (Brown, 1982). The difficulty in obtaining pure mouse acrosin precluded tests on its effect at higher concentrations, but it is most likely that these would have removed the zona pellucida more rapidly (Brown, 1982). The level of enzyme activity actually used in the present tests on the zona pellucida was approximately equivalent to that of ram acrosin used at 0.03 mg/ml, based upon a comparable hydrolysis of benzoylarginine-2-naphthylamide by the two

enzymes. At these comparable levels of activity ram acrosin took more than 18 h to remove the mouse zona pellucida (Brown, 1982) and in the present tests mouse acrosin took 6 h. This represents a more effective hydrolysis by the homologous enzyme, especially when its instability at pH 7.4 is taken into account. However, the zona pellucida could be subjected to much higher concentrations of acrosin *in vivo* (Brown, 1982).

The contrasting susceptibility of mouse and sheep zona pellucida to a pure homologous acrosin correlates with the widely different degrees of difficulty in carrying out successful in-vitro fertilization in these species. In the mouse, for example, in-vitro fertilization is readily achieved but in sheep, and other large domesticated species, it has not yet proved possible; failure has been due to the inability of the spermatozoon to penetrate the zona pellucida.

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