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# The Loss of a Low Molecular Weight Acrosin Inhibitor from Acrosomes during Capacitation (40232)

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Capacitation is a process which sperm of several mammalian species undergo in the female genital tract before achieving the ability to fertilize ova (1, 2). One step in fertilization is the penetration of the *zona pellucida* of the ovum by the sperm utilizing the acrosomal enzyme, acrosin (3–5), which can be inhibited by a number of synthetic or natural protease inhibitors (6–8). Acrosin activity is present in both epididymal and capacitated sperm of rabbit and boar but is very low in ejaculated sperm (9, 10). Zaneveld *et al.* (9) have proposed that an acrosin inhibitor present in seminal plasma is added to the sperm acrosomes during ejaculation, thereby inhibiting sperm acrosin. A number of inhibitors have been purified from several species including human (11) and boar (12, 13). They inhibit both acrosin and trypsin and are generally in 2 mol wt ranges, 6000 daltons and 11,000–13,000 daltons. Such inhibitors are found in the seminal plasma of semen and bound to the surface of the sperm acrosome. Zaneveld *et al.* (14) have shown one of these inhibitors is removed from the sperm surface during capacitation. Incubation of capacitated sperm with seminal plasma shows reappearance of the surface bound inhibitor.

The acrosin inhibitor we have found in rabbit and boar sperm is distinct from these inhibitors in its very low mol wt (2000 daltons or less) and its specificity of inhibition.

*Materials and methods.* Inhibitor assays were done using either boar sperm acrosin having a specific activity of 249 IU/mg purified by affinity chromatography using a modification of the method of Fritz *et al.* (15) or crystalline porcine pancreatic trypsin (Novo). The hydrolysis of benzoyl-arginine ethyl ester (BAEE) was measured at 25° in 0.05 *M* Tris–HCl buffer pH 8.0 at 253 nm. Two hundred microliters of a low molecular weight inhibitor solution was added to 200 μl of acrosin solution and then incubated at 25° for 5 min. This solution (0.2 ml) was then pipetted into 2.8 ml of 0.00025 *M* BAEE in 0.05 *M* Tris–HCl pH 8.0 in a cuvette. After quickly mixing the solution, the increase in optical density at 253 nm during the first 2 min of reaction was recorded using a Gilford model 240 spectrophotometer. One inhibition unit was the amount of inhibition that caused the reduction of BAEE hydrolysis by 0.001 $OD_{253}$ unit per min per ml of inhibitor, Burck *et al.* (16). Trypsin assays were done using the same technique and using as much as ten times the level of inhibitor.

Sperm samples were collected from boars of proven fertility by artificial vagina and pooled. Aliquots of 300 ml each containing $3 \times 10^9$ sperm were inseminated into sows on the second day of standing estrus or served as controls. Sperm were recovered from the uteri of sows sacrificed (with 50 ml of Lethal IV) 1, 2, 3, 4, and 6 hr after artificial insemination by excising the uterus and flushing each horn twice with 50 ml of $Ca^{++}$ free Ringer's solution. Sperm were collected from the flushings by centrifugation and washed three times with 50 ml $Ca^{++}$ free Ringers solution. Rabbit sperm were collected by artificial vagina from bucks of proven fertility and pooled. Aliquots of the pool containing $2 \times 10^8$ sperm were artificially inseminated into females which were given 75 IU of human chorionic gonadotrophin (HCG) IV. *In utero* incubated sperm were recovered 1, 2, 4 and 8 hr after insemination by excising the uterus of does sacrificed by cervical dislocation and flushing each horn twice with 5 ml of $Ca^{++}$ free Ringers solution. Sperm were collected by centrifugation and washed three times with $Ca^{++}$ free Ringers solution. *In vivo* capacitation assays were done in the rabbit by the method of Dukelow et al. (17).

Boar or rabbit sperm acrosomal extracts were obtained by either sonication of acrosomes isolated by Hyamine 2389 treatment or by direct NaOH extraction of whole washed sperm using the methods of Hartree



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and Srivastava (5). In one case the low molecular weight acrosin inhibitor was separated from the 6000 and 12,000 dalton inhibitors by chromatography of an acidified acrosomal extract on a 2.5 × 90 cm Sephadex G-50 (fine) column equilibrated with 0.01 *N* HCl at 5°. For the purpose of the work described in this paper the low mol wt acrosin inhibitor was routinely separated from the 6000 and 12,000 dalton inhibitors at pH 2.0 on an Amicon Ultrafiltration apparatus using a UM2 membrane which has a nominal mol wt cutoff of 2000 daltons. The UM2 filtrate, containing the low molecular weight inhibitor was freeze-dried and taken up in a minimum volume of 0.01 *N* HCl. This solution was further purified by chromatography on a 2.5 × 45 cm Sephadex G10 (fine) column equilibrated with 0.01 *N* HCl at 5° having a void volume of approximately 0.45 column volumes. Fractions were assayed for trypsin and acrosin inhibitor activity. Sperm counts were done using a hemocytometer.

To detect low molecular weight acrosin inhibitor in sow uterine washings, the sperm were first removed by centrifugation. The supernate was then adjusted to pH 2.0 with HCl and dialyzed against 0.01 *N* HCl using a UM2 membrane. Freeze-dried UM2 filtrates were again applied to a 2.5 × 45 cm Sephadex G10 (fine) column equilibrated with 0.01 *N* HCl at 5°.

*Results and discussion.* Chromatography of acidified boar sperm extracts on Sephadex G50 separated the acrosin (A) from the seminal plasma inhibitor (B). In addition a low molecular weight acrosin inhibitor (C) was found (Fig. 1). These two inhibitors could also be separated by ultrafiltration of acidified sperm extracts on Amicon UM2 membranes. The low mol wt inhibitor was found in the filtrate, while the higher mol wt seminal plasma inhibitors were completely retained by this membrane.

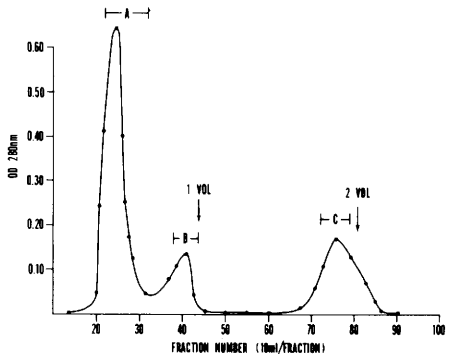


FIG. 1. Elution profile of acidified boar sperm extracts chromatographed on a 2.5 × 90 cm Sephadex G50 (Fine) column equilibrated in 0.01 *N* HCl. The profile shows the separation of (A) acrosin, (B) seminal plasma acrosin and trypsin inhibitor and (C) a low mol wt acrosin inhibitor.

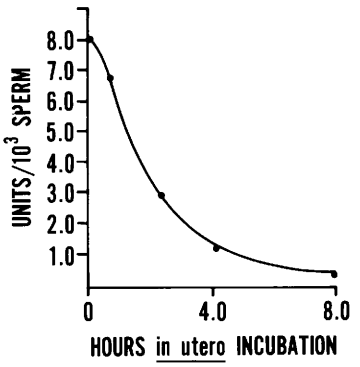


FIG. 2. Loss of acrosin inhibitor activity from rabbit acrosomes during *in utero* incubation of ejaculated sperm.

Concentrations of the partially purified low molecular weight inhibitor which completely inhibited one mg of boar acrosin (249 IU/mg) did not inhibit 1 mg of porcine trypsin using either BAEE or BAPNA as the substrates. Levels of low molecular weight inhibitor as much as ten times the above still showed no trypsin inhibition. From this we conclude the low molecular weight inhibitor is specific for acrosin even though we are working with an impure preparation which precludes establishing molar stoichiometry for the inhibition.

When the low molecular weight acrosin inhibitor fraction from rabbit sperm incubated *in utero* for varying lengths of time was assayed for the ability to inhibit the hydrolysis of BAEE by acrosin a nearly complete loss of this low molecular weight acrosin inhibitor activity was noted in 8 hr (Fig. 2). This loss of a specific low mol wt acrosin inhibitor over a period of time which coincides with the known *in utero* capacitation time of rabbit sperm is similar to the loss of the 6000 dalton inhibitor from the surface of the rabbit sperm acrosome observed by Zaneveld *et al.* (14).

When the UM2 filtrate fractions derived

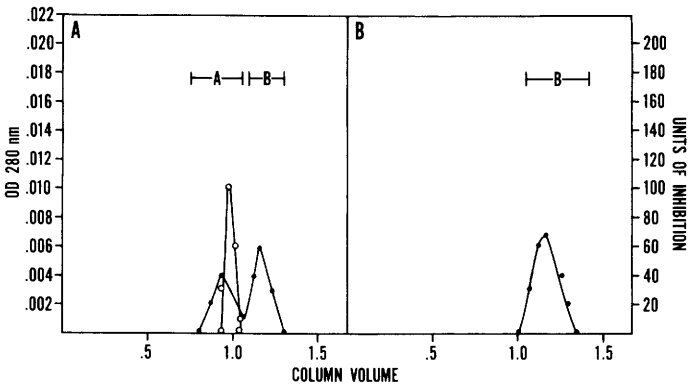


FIG. 3. Elution profile of UM2 filtrates of acrosomal extracts of (A) ejaculated rabbit sperm and (B) 8-hr *in utero* capacitated rabbit sperm on a 2.5 × 45 cm Sephadex G10 (Fine) column equilibrated with 0.01 *N* HCl at 5°. (●) Optical density at 280 nm and (○) acrosin inhibitor activity.

from washed ejaculated rabbit sperm and rabbit sperm incubated 8 hr *in utero* were chomatographed on Sephadex G10 in 0.01 N HCl, the elution profiles shown in Fig. 3 were obtained. Ejaculated rabbit sperm contain the low molecular weight acrosin inhibitor whereas 8 hr *in utero* incubated sperm acrosomes show a complete loss of this acrosin inhibitor in Fraction A. Capacitation of rabbit sperm incubated *in utero* for 8 hr was verified by direct insemination of sperm collected as previously described into the oviducts of rabbits using the method of Dukelow *et al.* (17). Fertilization was routinely observed when 8 hour *in utero* incubated rabbit sperm were artificially inseminated into the oviduct of does. If the 8 hr *in utero* incubated rabbit sperm were preincubated for 30 min with Fraction A and then inseminated into the oviduct, no fertilization occurred.

The low molecular weight acrosin inhibitor was also present in boar sperm acrosomal extracts. Experiments were designed to measure capacitation in the sow and to more fully examine the low mol wt inhibitor, since much larger sperm volumes could be utilized. Figure 4A shows the Sephadex G10 (Fine) elution profile of UM2 filtrates from acrosomal extracts of ejaculated boar sperm. The profile closely resembles that of rabbit sperm. Ejaculated boar sperm also contains a low molecular weight acrosin inhibitor. Boar sperm which have been incubated *in utero* in sows for two hours show a partial loss of the inhibitor in Fraction A while 4- and 6-hr *in utero* incubated sperm show a complete loss of the acrosin inhibitor in Fraction A (Figs. 4B, 4C, 4D). Capacitation time in the sow has not been established although Snider *et al.* (18) have observed changes in the lipid content of boar acrosomes at 90 min which they hypothesize to be involved with acrosomal membrane changes that precede acrosin activation. Our results indicate boar sperm would be capacitated after 3–4 hr *in utero* incubation.

Zaneveld *et al.* (19) have reported decapacitation of rabbit sperm by 30 minute incubation of capacitated rabbit sperm in seminal plasma. Figure 4E shows that 4-hr *in utero* incubated boar sperm partially regain the low molecular weight acrosin inhibitor activity in Fraction A when incubated with seminal plasma for one hour at 37°. Fraction A from boar sperm acrosomes will inhibit fertilization if preincubated with 8-hr *in utero* incubated rabbit sperm analogous to that seen for Fraction A from rabbit sperm indicating these inhibitors are not species specific.

UM2 filtrates of the washings of sow uteri which had been inseminated for four and six hours apparently contain two acrosin inhibitors (Fig. 5A) while flushings from a noninseminated estrus uterus lack the inhibitor found in Fraction X. Fraction X appears to have a higher molecular weight than Fraction A from sperm acrosomes and may represent Fraction A plus a uterine binding protein.

It is apparent that acrosin, the sperm enzyme responsible for penetration of the ovum *zona pellucida,* is inhibited by a low mol wt inhibitor in the acrosome which is removed from the acrosome during capacitation and passes into the uterus where it may complex

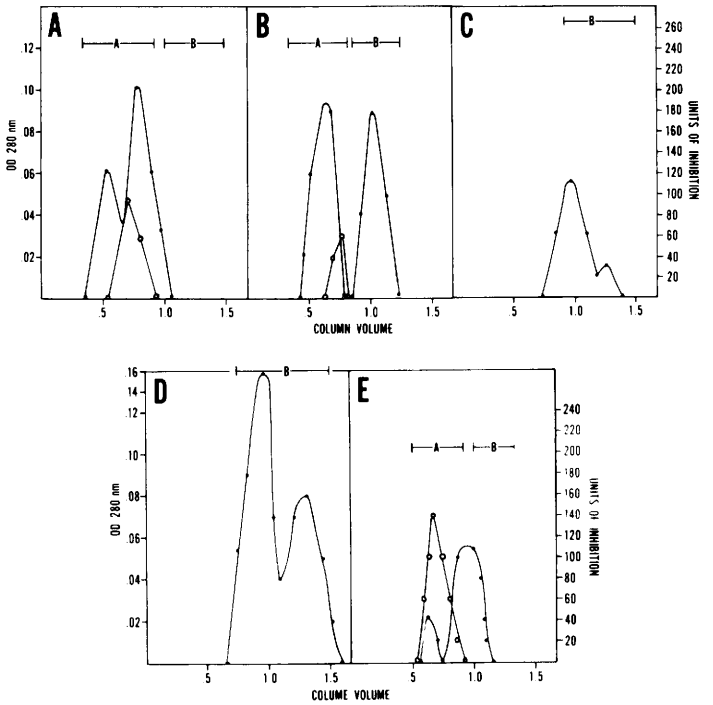


FIG. 4. Elution profile of UM2 filtrates of acrosomal extracts of (A) ejaculated boar sperm, (B) 2-hr, (C) 4-hr, (D) 6-hr *in utero incubated* boar sperm, and (E) 4-hr *in utero* incubated boar sperm which was incubated in boar seminal plasma for one hour at 37°. The 2.5 × 45 cm column of Sephadex G10 (Fine) column was equilibrated with 0.01 *N* HCl at 5°. (●) Optical density at 280 nm and (○) acrosin inhibitor activity.

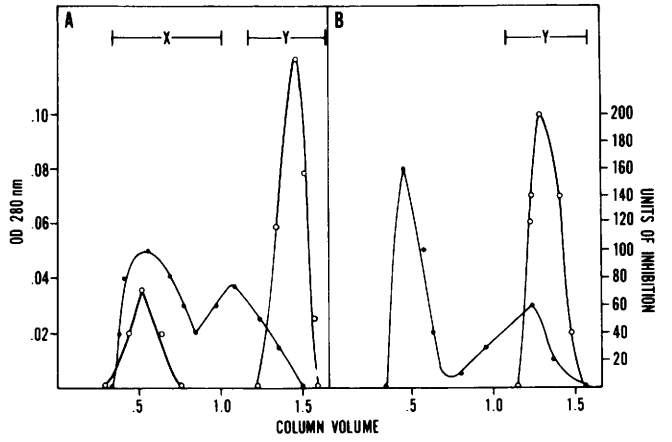


FIG. 5. Elution profile of UM2 filtrates of uterine flushings from (A) 4-hr inseminated and (B) non-inseminated estrus sow uteri on a 2.5 × 45 cm Sephadex G10 (Fine) column equilibrated with 0.01 *N* HCl at 5°. (●) Optical density at 280 nm and (○) acrosin inhibitor activity.

with another protein, thus preventing it from passing back through the acrosomal membrane. Polakoski *et al.* (20) have isolated proacrosin from the sperm acrosome which can be activated to yield acrosin. The low mol wt acrosin inhibitor may be an activation product of sperm acrosin analogous to those generated during the activation of other serine proteases.

*Summary.* Capacitation has been shown to be a series of reactions occurring in the uterus which enable the sperm to penetrate the outer

investments of the ova during fertilization. We have observed the loss of a low mol wt acrosin inhibitor from sperm acrosomes during *in utero* incubation of the sperm. Loss of this inhibitor is directly related to the sperm's ability to fertilize ova and is reversible by incubation of the capacitated sperm with seminal plasma. This low molecular weight acrosin inhibitor prevents fertilization in rabbits when preincubated with the capacitated rabbit sperm used to inseminate does.

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