AN ACROSIN-ACROSIN INHIBITOR COMPLEX IN EJACULATED BOAR SPERM

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Summary: Acrosin (a proteolytic enzyme) activity is high in extracts of *epididymal* sperm acrosomes but low in extracts of *ejaculated* sperm acrosomes. This lower activity from ejaculated sperm has previously been postulated as resulting from the reaction of a seminal plasma proteolytic enzyme inhibitor with acrosin in the acrosome. Evidence is presented which demonstrates that an acrosin-acrosin inhibitor complex exists in extracts of ejaculated boar sperm acrosomes. Upon incubation at pH 3 the complex dissociates and the free enzyme is demonstrable with the use of the synthetic substrate, benzyl arginine ethyl ester (BAEE). The increase in activity from incubation at pH 3 does not result from zymogen activation. Epididymal sperm have the ability to pick up the protease inhibitor from solution. The fact that there is no excess of free acrosin nor of free inhibitor in extracts of ejaculated sperm acrosomes is further evidence for the existance of an acrosin-acrosin inhibitor complex.

Introduction: Acrosin is a unique acrosomal protease (1) with properties in common with both plasmin and trypsin. Inhibition of acrosin with synthetic (2) or natural protease inhibitors (3,4) prevents fertilization both *in vivo* (2,3) and *in vitro* (4) presumably by blocking passage of sperm through the zona pellucida of the ovum (5). A previous report (6) has shown that acrosin activity is present in both rabbit epididymal and capacitated sperm, but is very low in ejaculated sperm. The high acrosin activity of epididymal sperm was lost upon incubation of intact sperm in seminal plasma (7). Zaneveld *et al.* (7) proposed that a seminal plasma acrosin inhibitor was added to acrosin during ejaculation.

Kunitz and Northrop (8) demonstrated that some protease-protease inhibitor complexes dissociate under acidic conditions. This report describes experiments that show the existance of an acrosin-acrosin inhibitor complex in extracts of acrosomes.

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Methods: Sperm obtained from boar ejaculates were washed and the acrosomes removed by the method of Hartree and Srivastava (9) as modified by Polakoski, Zaneveld, and Williams (10). Epididymal boar and rabbit sperm were flushed from the epididymides and the acrosomes removed by detergent (10). Acrosomal extracts (1 mg protein/ml) were suspended in 0.05 M borate buffer at pH 7.6 containing 0.05 M $CaCl_2$ centrifuged and 0.1 ml added to 2.9 ml 0.05 M Tris-HCl buffer containing 0.0017 M BAEE (Mann) (11). Acidic incubation of the above extracts was done by adjusting to pH 3.0 with 1 M HCl, incubating at this pH for a minimum of 5 min and then adding 0.1 ml to the BAEE solution at pH 8.0 and observing the change of optical density at 253 mμ (10). Addition of this small aliquot of enzyme solution to the substrate prevents formation of the inactive complex.

Results and Discussion: Curve A in Fig. 1 shows the acrosin activity in extracts of epididymal boar sperm acrosomes after preincubation at pH 7.6. Curve B in Fig. 1 shows the acrosin activity in extracts of epididymal boar

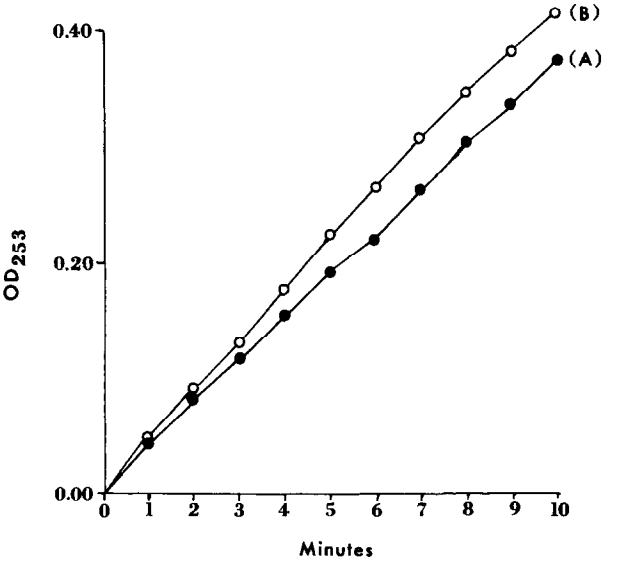

Fig. 1. Acrosin activity of an epididymal boar acrosomal extract incubated in succession as follows:

A (●) 1st Incubation, pH 7.6, assay at pH 8.0
B (o) 2nd Incubation, pH 3.0, assay at pH 8.0

sperm acrosomes after the extracts were adjusted to pH 3.0 and preincubated prior to the assay. Curves A and B in Fig. 2 show the acrosin activity in extracts of ejaculated boar sperm acrosomes after similar treatment. One explanation for the increase in acrosin activity shown in Curve B in Fig. 2 is dissociation at pH 3.0 of an acrosin-acrosin inhibitor complex. A second possibility is that acidic pH activated a zymogen form of acrosin. To examine this second possibility the ejaculated acrosomal extract after incubation at pH 3 was again incubated at pH 7.6 and tested for acrosin activity. As can be seen in Curve C of Fig. 2, essentially no acrosin activity resulted, indicating that either denaturation of the enzyme occurred or that an inhibitor complex was formed. Curve D of Fig. 2 shows the result of again incubating the extract at pH 3.0, the high acrosin activity indicating that the enzyme was not denatured. These results strongly suggest the presence of an acrosin-acrosin inhibitor complex in extracts of ejaculated sperm acrosomes and very

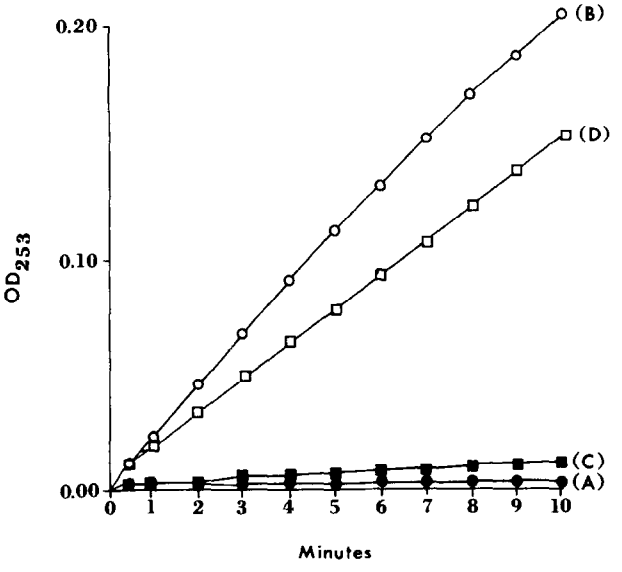

Fig. 2. Acrosin activity of an ejaculated boar acrosomal extract incubated in succession as follows:

A (●) 1st Incubation, pH 7.6, assay at pH 8.0
B (○) 2nd Incubation, pH 3.0, assay at pH 8.0
C (■) 3rd Incubation, pH 7.6, assay at pH 8.0
D (□) 4th Incubation, pH 3.0, assay at pH 8.0

likely in the acrosome. The purification and characterization of the inhibitor will be published elsewhere (11).

Since the major difference between epididymal sperm and ejaculated sperm results from a brief incubation of the sperm in the seminal plasma and since we have available a purified acrosin inhibitor from boar seminal plasma (12), means were available to determine if epididymal sperm adsorbed the purified inhibitor. Six mg of purified acrosin inhibitor was incubated for 15 min with 2 x 10^9 washed epididymal rabbit sperm in 50 ml of Krebs Ringer Phosphate and the acrosomes were removed and extracted (10). Lines A and B of Fig. 3 show the acrosin activity of the extracts after preincubation at pH 8 (A) and pH 3 (B) as described for previous experiments. The control without inhibitor was identical to line B of Fig. 3. From these results it appears the boar inhibitor is taken up by epididymal rabbit sperm.

The possibility exists that the inhibitor is bound non-specifically to

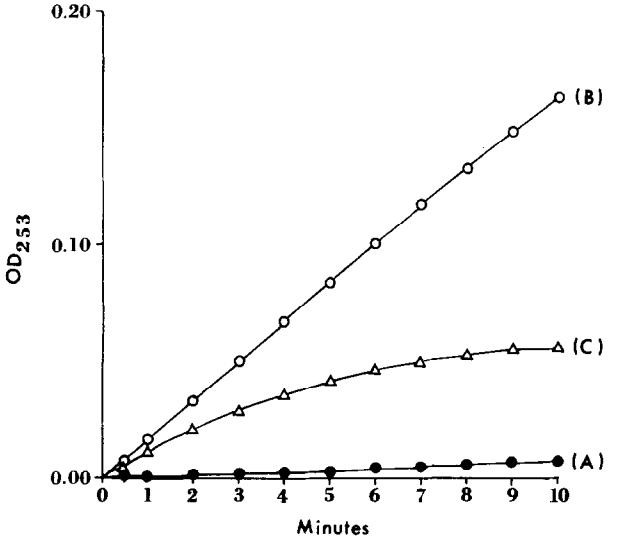

Fig. 3. Acrosin activity of an extract of epididymal rabbit sperm that were previously incubated with purified boar seminal plasma acrosin inhibitor.

A (●) 1st Incubation, pH 8.0, assay at pH 8.0
B (○) 2nd Incubation was done at 3.0, assay at pH 8.0
C (△) Same as B but in presence of 2.5 µg purified boar seminal plasma acrosin inhibitor

the exterior of the acrosome. If this were true, one would expect either an excess of enzyme or of inhibitor in the extracts of ejaculated sperm acrosomes. The insignificant amount of activity (line A in Fig. 2) after incubation at pH 7.6 suggest there is no excess of enzyme. To determine the effect of an excess of inhibitor, 2.5 μg of purified boar trypsin inhibitor was added to the substrate mixture and the assay carried out after preincubation at pH 3.0. The hyperbolic curve that results (line C of Fig. 3) indicates the excess of inhibitor competes with the substrate for the enzyme. Similar results are obtained if the inhibitor is added to either substrate or incubation mixture of the ejaculated extracts. The straight line obtained after incubation of the initial extract at pH 3.0 (line B in Fig. 1,2 and 3) without added inhibitor suggest there is no excess of inhibitor in these extracts.

A more extensive study and discussion on extracts of sperm from 11 species will be reported elsewhere (13). It appears the occurrence of an acrosin-acrosin inhibitor complex in acrosomes of ejaculated sperm is a general phenomenon and not unique for extracts of boar sperm acrosomes. The results reported herein and by Zaneveld *et al.* (7) support the hypothesis that acrosin inhibitor from seminal plasma is added to acrosin at the time of ejaculation (14).

The high acrosin activity at alkaline pH in extracts of capacitated sperm acrosomes (6) and the fact that trypsin inhibitors prevent fertilization by capacitated sperm *in vivo* (2,3) and *in vitro* (4) indicates the inhibitor is removed during capacitation in the female tract.

The physiological advantage of this apparent regulatory role of the inhibitor is presently only a subject for speculation and may be clarified only after a better understanding of the biochemical processes of fertilization in general is achieved.

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