Boar Acrosin

I. PURIFICATION AND PRELIMINARY CHARACTERIZATION OF A PROTEINASE FROM BOAR SPERM ACROSOMES*

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SUMMARY

Acrosin is a proteolytic enzyme used by sperm to digest a path through the zona pellucida of the ovum. In ejaculated sperm it is inactivated by a proteinase inhibitor from seminal plasma that also inhibits trypsin. This inhibitor is removed or inactivated during the residence in the female reproductive tract as a part of the capacitation process.

The boar acrosin-inhibitor complex was partially purified using DEAE-cellulose chromatography. The complex was dissociated and the acrosin obtained in a highly purified form by the use of Sephadex gel filtration at pH 3.0. The final preparations were judged to be homogeneous on the basis of chromatographic and electrophoretic criteria and showed a molecular weight of 30,000. Acrosin has a temperature optimum of 53°, an esterase optimum at pH 8.5, and a proteolytic optimum on azocasein at pH 8.7. The enzyme is quite stable at pH 3.0 but undergoes destruction at slightly alkaline pH. Purified acrosin is both activated and stabilized by calcium ions; however, it is not stable to freezing nor to freeze-drying and is inactivated by urea.

Previous reports have shown that acrosin activity is present in both epididymal and capacitated sperm, but the activity is low or absent in ejaculated sperm due to the presence of a seminal plasma proteinase inhibitor (7, 8).

This report describes the separation of boar acrosin from the seminal plasma proteinase inhibitor and the purification of boar acrosin to homogeneity as judged by sodium dodecyl sulfate gel electrophoresis. Preliminary characterization of this enzyme is also described. A preliminary report was presented previously (9). Fink et al. (10) have published the partial purification of boar acrosin using affinity column procedures.

EXPERIMENTAL PROCEDURES

Materials—Diethylaminoethylcellulose-52 was purchased from Whatman. Sephadex G-200, Sephadex G-100, Sephadex G-75, and deacetau blue were obtained from Pharmacia. Tris-(hydroxymethyl)-aminomethane (ultra pure); urea (ultra pure) and benzoylarginine ethyl ester were from Mann. Dimethylidichlorosilane was a product of Bio-Rad Laboratories. Sodium dodecyl sulphate was from BDH Chemicals, Ltd. Pepsin was from California Biochemicals Co., and porcine pancreatic trypsin was a gift from Dr. J. Travis of the University of Georgia. Lysozyme was purchased from Worthington; Pentex supplied the bovine serum albumin and the ovalbumin. The carbonic anhydrase from bovine erythrocytes was purchased from BioResearch, Inc. Hyamine 2389 and Triton X-100 were obtained from Rohm and Haas, and 1,4-piperazine-bis-(ethanesulfonic acid) sodium salt was from Calbiochem. Solutions used were made with glass-distilled water and reagent grade chemicals unless otherwise specified.

Starting Material—Boar semen was centrifuged at 3200 rpm for 1 min and the supernatant was used as the starting material.

Esterase Assays—The esterase activity of acid and pancreatic trypsin was determined by the method of Schwert and Takanaka (12). Unless otherwise specified, the reaction mixture contained 498 μg of BAEI in 2.9 ml of 0.15 M Tris buffer, pH 8.0, plus 0.05 M CaCl₂. The enzyme solution was added in 0.1 ml, and the change in absorbance at 253 nm was recorded every 30 s at 23° with a Gilford model 240 spectrophotometer. A unit of acrosin activity was defined as an absorbance change of 0.001 at 253 nm in 30 s at 23°.
change of 1.0 optical density (O.D.) unit per min and specific activity as acrosin units per mg of protein. Protein was estimated by measuring absorption at 280 nm assuming 1 mg of protein per ml exhibits an O.D. of 1.0 at 280 nm in a 1-cm light path. The validity of this assumption was confirmed by comparing values to protein estimated by the method of Lowry et al. (13) using bovine serum albumin as a standard.

The inhibitor was assayed by incubation with porcine pancreatic trypsin in 0.4 ml of buffer at 23° for at least 10 min, and the resulting esterase activity was determined by using 0.1 ml of the incubation mixture. Reproducible results were obtained provided the inhibitor concentration was adjusted so that no more than 70% of the trypsin was inhibited. One inhibitor unit is that amount of inhibitor that reduced BAEE hydrolysis by 1.0 A_{283} unit per min (14).

**Proteolytic Assays**—Assays were designed for a total volume of 2 ml. One milliliter of a 1% azocasein solution in 0.05 M Tris-HCl at pH 8 and an appropriate amount of buffer were brought to 37° in a waterbath for at least 5 min. Enzyme solutions were added at 30-s intervals, and after appropriate incubation stopped by addition of 2 ml of 10% trichloroacetic acid. After thorough mixing and filtration through Whatman No. 1 filter paper, the solubilized dye in the filtrate was read at 366 nm and corrected for control values.

**Enzyme Purification**—Purification was accomplished by chromatography on columns using DEAE-cellulose and Sephadex support media. The glass columns were siliconized prior to use, and all chromatography was performed at 4° using plastic test tubes for collecting the eluate to avoid enzyme absorption on glass. The DEAE-52 column (1.6 × 15 cm) was packed and equilibrated in 0.05 M Tris-HCl buffer, pH 8.0. The Sephadex G-100 column (5 × 45 cm) and the Sephadex G-75 superfine column (2.5 × 95 cm) were packed and equilibrated in 0.001 M HCl, pH 3.0, containing 0.1 M NaCl. The height of the gel bed in the columns remained constant throughout the experiment. Molecular weight estimations were performed on the Sephadex G-75 superfine column by the method of Andrews (15). The void volume of the column was determined with dextran blue. Values used for the molecular weights of the reference proteins were as follows: ovalbumin, 42,000; pepsin, 35,000; carbonic anhydrase, 29,000; trypsin, 23,300; and lysozyme, 14,300.

**Electrophoresis**—Sodium dodecyl sulfate gel electrophoresis was carried out by the method described by Weber and Osborn (16), omitting the final dialysis step in the preparation of the samples. Bovine serum albumin, ovalbumin, pepsin, porcine pancreatic trypsin, and lysozyme were used as reference proteins. The gels were stained with 0.25% Coomassie brilliant blue in a 50% methanol and acetic acid solution (45:46) and destained in a Hoefer diffusion destainer. Analytical gel electrophoresis was performed in glass columns (5 × 52 mm) using 7.5% acrylamide at pH 4.3 as described by Brewer and Ashworth (17). The sample solution was mixed with a drop of glycerol and layered on top of the running gel, thus omitting the upper gel. A current of 4 mA per gel was applied until the tracking dye moved 40 mm into the gel. Reference gels were stained as described above, and corresponding gels were sliced with a Brinkmann gel slicer into 1-mm slices for assay of proteolytic activity. The slices were placed separately on Kodak projector slide photographic plates, approximately 100 μl of 0.05 M Tris-HCl buffer at pH 8.0 was placed on the individual gel slices, and the plates incubated in a humidified chamber at 37° for 6 hours. After the incubation, the plates were removed and washed with distilled water and observed for gelatin digestion.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (units/mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction of acrosomes</td>
<td>23.7</td>
<td>70.6</td>
<td>3.0</td>
<td>1</td>
<td>100</td>
</tr>
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<td>56.3</td>
<td>9.0</td>
<td>3</td>
<td>79</td>
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<tr>
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<td>96.0</td>
<td>32</td>
<td>75</td>
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<tr>
<td>Sephadex G-75 (superfine) column</td>
<td>0.39</td>
<td>40.2</td>
<td>104</td>
<td>35</td>
<td>58</td>
</tr>
</tbody>
</table>

* The enzyme was completely inhibited with a natural proteinase inhibitor and no activity was demonstrable until the complex was dissociated at pH 3.0 prior to assay at pH 8.0 (8).

| Results | Purification of Boar Acrosin | 8179 |

A purification scheme for isolating boar acrosin is illustrated in Table I. Because of the presence of the proteinase inhibitor, there was no activity in either the crude extract or the material from the DEAE column chromatography unless the extract was incubated at pH 3.0 prior to assay at pH 8.0. The acrosin-inhibitor complex has a positive charge at pH 8.0 and is not retained on the DEAE-cellulose column. Void volume fractions containing the complex were pooled and concentrated from 30 to 9.9 ml by placing the enzyme solution in dialysis tubing and adding dry Sephadex G-200 to the outside of the bag at 4°. The solution was adjusted to pH 2.0 with 1 M HCl and incubated for 3 hours at 4° to allow for complete dissociation of the acrosin-inhibitor complex. When this solution was applied to the Sephadex G-100 column, acrosin activity was normally eluted in a single peak. Some chromatographs showed two peaks of acrosin activity, a second peak eluting earlier. Rechromatography of this earlier peak resulted in the single acrosin elution band, suggesting a possible monomer-dimer relationship.

Elution of the proteinase inhibitor also occurs in a single band following acrosin. Because of the small amount of protein and the absence of trypthran in the inhibitor (18) the optical densities were read at 220 nm. Tubes containing activity were pooled and concentrated on a Diaflow ultrafilter (Amicon) equipped with a UM-02 membrane. The assay for the proteinase inhibitor showed 4.2 units. The further purification and characterization of the inhibitor will be reported in a separate publication. Tubes containing acrosin activity were pooled and concentrated from 68 to 10 ml with dry Sephadex G-200, adjusted to pH 3.0, and chromatographed on the equilibrated Sephadex G-75 column. The elution profile of this column is shown in Fig. 1. The constant specific activity across the enzyme peak was 148 units per mg of protein when protein was determined by the Lowry method (13).

**Sodium Dodecyl Sulfate Gel Electrophoresis**

To evaluate further the purity of the acrosin preparation, it was dialyzed against 0.001 M HCl at 4° for 20 hours, freeze-dried, and concentrated on a Diaflow ultrafilter (Amicon) equipped with a UM-02 membrane. The assay for the proteinase inhibitor showed 4.2 units. The further purification and characterization of the inhibitor will be reported in a separate publication. Tubes containing acrosin activity were pooled and concentrated from 68 to 10 ml with dry Sephadex G-200, adjusted to pH 3.0, and chromatographed on the equilibrated Sephadex G-75 column. The elution profile of this column is shown in Fig. 1. The constant specific activity across the enzyme peak was 148 units per mg of protein when protein was determined by the Lowry method (13).

* The acrosin-inhibitor complex dissociates under acidic conditions and when the dissociated complex is assayed with BAEE the large excess of substrate displaces the inhibitor on the enzyme's active site and, therefore, initial activity can be determined (8).
and suspended in 0.1 M phosphate buffer, pH 7.0, with 1% sodium dodecyl sulfate and 1% mercaptoethanol. After a 2-hour incubation at 37°, it was subjected to electrophoresis in the standard sodium dodecyl sulfate gel electrophoresis system as described by Weber and Osborn (16). Only one major band of protein was detected in the purified enzyme solution.

**Molecular Weight Estimation**

Comparison of the enzyme band in the sodium dodecyl sulfate gels to that of the reference proteins gave an indicated molecular weight of 30,000, a value in agreement with the 30,000 obtained from the gel filtration determination. The standards, ovalbumin, bovine serum albumin, pepsin, pancreatic trypsin, and lysozyme, were each run separately with 4 mg of each protein dissolved in 2.0 ml of buffer.

**Gel Electrophoresis**

Gel electrophoresis was performed using 7.5% acrylamide gel electrophoresis at pH 4.3. Due to the instability of the purified protein during concentration procedures (see “Stability Studies”), volume limitations allowed only 2 to 3 µg of purified active enzyme to be applied to the gel. Although this amount of protein gave only one faint band with Coomassie blue staining, an evaluation of contaminating protease activity could be carried out with the photographic plate technique. The gel was sliced and the disks placed on a photographic plate. Two consecutive slices contained proteolytic activity after a 6-hour incubation. The photographic plate technique was highly sensitive, since digestion of the gelatin by 4 ng of porcine trypsin occurred in 1 hour. The demonstration of a lack of contaminating protease activity was important for specificity studies (19).

**Properties of Boar Acrosin**

In all of the following characterization studies (except where noted) approximately 0.5 to 1.0 µg of the enzyme was used in the assay. In each case only plasticware was used because the purified acrosin is adsorbed on glass.

**pH Optimum**—The pH dependence of acrosin-esterase activity was determined using Pipes and Tris buffer solutions, each containing 0.05 M CaCl₂. Esterase activity was minimal at pH 5.5, rising sharply to the optimum at pH 8.5. A similar decline occurred at higher pH values. The proteolytic optimum for acrosin in azocasein was found to be pH 8.7.

**Effect of Temperature on Proteolytic Activity**—The results presented in Fig. 2 show that the optimum temperature of proteinase activity against azocasein was 53°.

**Effect of Calcium Ions**—The effect of calcium ions on the hydrolysis of BAEE by acrosin is shown in Fig. 3. The activities reported were for the first minute of hydrolysis, and an approximate 2-fold increase in activity resulted from 0.05 M calcium ion.

**Stability Studies**—Fig. 4 shows the stabilizing effects of calcium ions. Line A shows that acrosin is stable at pH 3.0 in the absence of calcium. Curve B shows acrosin to be relatively stable at pH 8.0 in the presence of 0.05 M Ca²⁺ after an initial slight loss of activity, while Curve C illustrates the instability of acrosin at pH 8.0 without calcium.

The inactivation of acrosin by urea is shown in Fig. 5. An approximate 10% loss in activity occurred even at 0.05 M urea with 50% loss in activity occurring at 2.7 M urea. Porcine pancreatic trypsin retained over 95% of its activity even in 5 M urea which is in good agreement with Harris (20) and Kafatos et al. (21).

The temperature stability of acrosin was determined by in-

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**Fig. 1.** Sephadex G-75 superfine column (2.5 × 90 cm) chromatography of the acrosin obtained from the Sephadex G-100 column. Flow rate was 14 ml per hour and 3.7-ml fractions were collected. ●—●, absorbance at 280 nm; ○—○, acrosin activity; □—□, specific activity of acrosin.

**Fig. 2.** Effect of temperature on the proteolytic activity of acrosin using azocasein as substrate. The incubation mixtures contained 0.05 M Tris-HCl buffer, pH 8.0, and were preincubated for 10 min prior to the addition of enzyme. The reaction was carried out for 30 min at the various temperatures and was stopped with 2.0 ml of 10% trichloroacetic acid and the tubes then cooled to 0°.

**Fig. 3.** Effect of calcium ions on the activity of acrosin on BAEE.
The incubations were performed at 23°. The incubation of the enzyme at various temperatures in 0.001 M HCl and 0.05 M NaCl was required temperature for 10 min and cooled immediately in an ice bath. Incubation of the enzyme in the absence of substrate at all temperatures above 37° gave major destruction of activity. Half of the activity was destroyed at 55°.

Acrosin obtained from either the Sephadex G-100 or G-75 column eluates dialyzed overnight against 0.001 M HCl gave no appreciable loss in enzymatic activity. Freeze-drying of the enzyme solutions showed a loss of 75 to 80% activity. The purified enzyme solution was also unstable to freezing and thawing. When the enzyme in 0.001 M HCl and 0.1 M NaCl was frozen in an acetone Dry-Ice bath and thawed at 25° three consecutive times, the activity remaining was 70, 41, and 20%, respectively. The enzyme lost only 5 to 10% of its activity upon storage for 3 weeks at 4° in the same solution.

**Fig. 4.** Stabilizing effect of calcium ions on porcine acrosin. A, enzyme in 0.001 M HCl, pH 3.0; B, enzyme in 0.05 M Tris-HCl and 0.05 M Ca²⁺, pH 8.0; C, enzyme in 0.05 M Tris-HCl, pH 8.0. The incubations were performed at 23°.

**Fig. 5.** Comparison of the effect of urea on acrosin and porcine trypsin activity. In each assay the enzyme was added to a fresh mixture of substrate and the desired concentration of urea with no preincubation of urea and enzyme in the assay cuvette. Initial velocity of the enzymatic reaction was measured. ■—■, acrosin; ○—○, trypsin.

The purified enzyme obtained at various temperatures in 0.001 M HCl and 0.1 M NaCl, pH 3.0. The enzyme solution was kept at the required temperature for 10 min and cooled immediately in an ice bath. Incubation of the enzyme in the absence of substrate at all temperatures above 37° gave major destruction of activity. Half of the activity was destroyed at 55°.

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**DISCUSSION**

The presented purification scheme gives a relatively pure preparation of acrosin by a few chromatography procedures with yields of better than 50%. This high recovery is of critical importance when one considers the difficulty of obtaining starting material and the instability of the purified enzyme.

The initial discarding of the material that precipitates in the light centrifugation of the semen is an important step, for it has allowed an increase in specific activity of the acrosomal extract from less than 0.6 to 3.0. The final acrosin preparation appears to be homogeneous as judged by the single protein component in the sodium dodecyl sulfate gel electrophoresis, a single symmetrical protein peak of uniform activity on gel filtration, the high specific activity of the most purified fraction and the lack of contaminating proteinase activity. Further characterization has been limited by the small amount of enzyme that is presently available. The present study constitutes the first report of obtaining a highly purified sperm acrosomal enzyme.

The specific activity of 104 reported in the present study as well as the specific activity of 18 we reported for the partially purified rabbit epididymal sperm acrosin (5) contrast with the results of Stambaugh and Buckley. Although they claim isolation of the enzyme (22), their most potent enzyme preparation had a specific activity of 1.46 (23).

Fink et al. (24) have partially purified boar acrosin with the use of affinity chromatography and have shown that it is strongly absorbed on glass surfaces in neutral or weakly basic solutions. Unfortunately, no comparison can be made since their criterion of purity is a specific activity of 5 on N-α-benzoyl-DL-arginine-p-nitroanilide, which was not tested as a substrate for our purified enzyme.

Due to the sensitivity of the photographic plate technique used in this study, the presence of contaminating proteinases was easily excluded. This exclusion was essential for specificity studies (19) since the multiple proteolytic activities present in sperm acrosomes from a wide range of species have been well documented (25–39). If enough detailed information about this enzyme can be obtained, a completely specific active site-directed reagent that could be used for contraceptive purposes may be synthesized.

We have previously found the molecular weight of rabbit acrosin to be 35,000 on the basis of Sephadex G-100 column chromatography according to the Andrews method (15), but the enzyme had a molecular weight of 27,300 when determined by sodium dodecyl sulfate gel electrophoresis. At that time we proposed the enzyme was in a possible dimeric form (5). The present results gave a molecular weight of 30,000 by both gel filtration and sodium dodecyl sulfate gel electrophoresis, with higher molecular weights only occasionally being seen in the Sephadex G-100 step. This would indicate that if boar acrosin does form a dimer under certain conditions the enzyme is probably active in both the monomeric and dimeric forms. The molecular weight of the monomeric form is comparable to the molecular weight of 30,000 reported for human acrosin (40).

Optima of pH 8.0 with BAEE and pH 8.7 with azocasein are slightly more alkaline than that obtained with purified rabbit acrosin (5). It should be noted that the increase in activity that occurred between pH 5.5 and 7.5 may result from the ionization of an active site histidine (41).

The purified acrosin suffers an irreversible loss of activity upon freezing or freeze-drying. Calcium ions increased the activity of trypsin by approximately 25% (42) and also caused a conformational change in the trypsin molecule, resulting in a more compact structure that is less sensitive to autolysis (43). Acrosin activity is increased by about 100% with calcium ion, and the activity is also partially protected at pH 8.0 by 0.05 M calcium ion. The instability without calcium even in plastic test tubes at pH 8.0 appears to follow second order kinetics, as does that of trypsin (44), but the activity is lost at a much faster rate than that of trypsin. This loss of activity occurs at a reduced rate at lower temperatures and more dilute enzyme solutions.*

* K. L. Polakoski, unpublished data.
It is apparent that trypsin is more stable in the presence of urea than acrosin, and this could be due to a difference in cystine residues in the respective molecules. Acrosin is similar to coonase in this instability, and it has been shown that coonase (21) has one cystine compared to three for trypsin (45). Unfortunately, not enough of the purified acrosin has been available for amino acid composition studies, but it is tempting to speculate that the tertiary structure of acrosin is primarily stabilized by hydrogen bonds that would be disrupted by urea. This suggests that acrosin is a less compact molecule that would more readily undergo autodigestion.

Meizel (46) has reported that there may be a zymogen form of an acrosin-like enzyme from rabbit testes. We have found no evidence for a zymogen form of acrosin in either ejaculated or epididymal sperm (8) although it is possible the enzyme is produced in the testes as a zymogen that is activated by the time the sperm reaches the epididymis.

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