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 decaamyl blue were obtained from Pharmacia. Tris-(hydroxymethyl)-aminnomethane (ultra pure); urea (ultra pure) and benzoylarginine ethyl ester were from Mann. Dimethyl dicarbamyl sulphone 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 was from Schwarz 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 3,000 rpm at 23° for 1 min and the upper nine-tenths of the material was processed by the method of Srivastava et al. (11) as modified by Polakoski et al. (5).

Esterase Assays—The esterase activity of acrosin and pancreatic trypsin was determined by the method of Schwert and Takanaka (12). Unless otherwise specified, the reaction mixture contained 498 µg of BAEE 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 read every 30 s at 23° with a Gilford model 240 spectrophotometer. A unit of acrosin activity was arbitrarily defined as an absorbance change of 0.0001 at 253 nm per min.

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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 A285 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- to 60-min 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 adsorption on glass. The DEAE-52 column (1.8 x 15 cm) was packed and equilibrated in 0.05 M Tris-HCl buffer, pH 8.0. The Sephadex G-100 column (5 x 45 cm) and the Sephadex G-75 superfine column (2.6 x 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, 22,000; pepsin, 36,000; 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 50% methanol and acetic acid solution (45:46) and dehydrated in a Hoffer diffusion destainer. Analytical gel electrophoresis was performed in glass columns (5 x 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.

Elution of the proteinase inhibitor also occurs in a single band following acrosin. Because of the small amount of protein and the absence of tryptophan in the inhibitor (18) the optical densities were read at 220 nm. Tubes containing activity 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 3.0 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.

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.* A 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 3.0 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.

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 chromatographed at 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,
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. O---O, absorbance at 280 nm; O-U, acrosin activity; ■-■, specific activity of acrosin.

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 temperature stability of acrosin was determined by in-
The incubations were performed at 23°.

Storage for 3 weeks at 4° in the same solution.

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 spectrophotometric 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.

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 (40)

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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 coconase in this instability, and it has been shown that coconase (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 acrosin in either ejaculated or epididymal sperm (8) although it is possible the enzyme is undergoing autodigestion. 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 partially autodigested.

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