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Purification of a Proteolytic Enzyme From Rabbit Acrosomes[1]

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A proteolytic enzyme assigned the name acrosin was purified 250-fold from the acrosomes of epididymal rabbit spermatozoa by DEAE and Sephadex chromatography. The acrosin preparations were of high purity as judged by the specific activity of 15 and since SDS gel electrophoresis showed only one major component. In the purified form acrosin is unstable above pH 6 even at low temperatures, but stable if lyophilized. The molecular weight of acrosin was estimated to be 55,000 by Sephadex chromatography. The enzyme most likely exists as a dimer since treatment with SDS and mercaptoethanol and subsequent molecular weight determination by polyacrylamide gel electrophoresis gave a subunit of molecular weight 27,300.

We have obtained a highly purified preparation of a proteolytic enzyme from rabbit sperm acrosomes that has certain properties in common with pancreatic trypsin and plasmin yet is different from both (Zaneveld, Polakoski, and Williams, 1971). The name, acrosin,[2] was proposed for this unique enzyme essential for fertilization (Zaneveld, Robertson, and Williams, 1970) in conformity with common usage for proteases where the prefix indicates the source of the enzyme followed by -in (Christensen and MacLeod, 1945). Stambaugh and Buckley (1971) suggested the name acrozonase. Yamane (1935a, b) first reported the presence of a proteolytic enzyme in mammalian spermatozoa. Buruiana (1956) showed that avian sperm are high in trypsin-like activity but low in hyaluronidase, whereas the reverse was true for mammalian spermatozoa. Rabbit sperm were an exception since both trypsin and hyaluronidase activities were present. Hartree and Srivastava (1965) and Srivastava, Adams and Hartree (1965) prepared acrosomal extracts from ram sperm that removed the zona pellucida from rabbit ova. Using essentially a modification of their technique, Stambaugh and Buckley (1969) showed that extracts of epididymal sperm removed the zona pellucida and that a trypsin-like enzyme (acrosin) located in the acrosomes was responsible. These results were confirmed and extended by Zaneveld, Srivastava, and Williams (1969), and Zaneveld and Williams (1970) who showed that ejaculated rabbit sperm acrosomes also contained acrosin inactivated by a trypsin inhibitor from seminal plasma. Because epididymal and capacitated spermatozoa had high acrosin activity, it was suggested that the trypsin inhibitor was added to epididymal sperm during ejaculation and removed during capacitation. Additional evidence for this hypothesis was provided by showing that epididymal sperm lose most of their proteolytic activity by exposure to seminal plasma (Zaneveld, Srivastava, and Williams, 1970). A similar suggestion was made earlier by Waldschmidt, Hoffmann, and Karg (1966) based on their observations concerning the proteolytic activity of whole bull sperm. Fertilization *in vitro* by capacitated sperm was inhibited by soybean or ovo-

[1] The Enzyme Commission of the International Union of Biochemistry has recently assigned the name, acrosomal proteinase, to a trypsin-like enzyme from the sperm acrosome.

[2] Unfortunately the more euphonious name, acrosomin, has been used for a polysaccharide (Clermont and Leblond, 1955). Trivial names for proteolytic enzymes are derived from the source plus "in" (Christensen and MacLeod, 1945; Enzyme Nomenclature, 1964).

mucoid trypsin inhibitors (Stambaugh, Brackett, and Mastroianni, 1969), or *in vivo*, using pancreatic trypsin inhibitor or a trypsin-inhibitor partially purified from seminal plasma (Zaneveld, Robertson, Kessler, and Williams, 1970) showing that acrosin is essential for fertilization. Fertilization can also be inhibited using synthetic trypsin inhibitors, such as tosyl lysine chloromethyl ketone (TLCK) (Shaw, 1970), either by addition to ejaculated or capacitated sperm or by deposition in the vagina (Zaneveld, Robertson, and Williams, 1970).

Stambaugh and Buckley (1969) reported that a trypsin-like enzyme in crude acrosomal extracts is complexed with hyaluronidase, the complex having a molecular weight of 59,000. In this article we describe the partial purification of acrosin including molecular weight estimations using two methods. The properties of acrosin are discussed in a separate paper (Zaneveld, Polakoski, and Williams, 1971).

MATERIALS AND METHODS

Preparation of Acrosomal Extracts

Epididymal rabbit spermatozoa were obtained by inserting a 25 g needle into the vas deferens and forcing Krebs Ringer Phosphate (KRP) buffer through the epididymis after having severed the caudal end. The sperm were washed several times with KRP and treated with 0.075% Triton X-100 (Mann Research) and 0.075% Hyamine 2389 (British Drug Houses, Ltd.) for 90 min at 37C, a procedure that causes the removal of the acrosomes from sperm (Hartree and Srivastava, 1965). The sperm mixture was centrifuged, the supernatant solution treated with alcohol and the precipitated material dialyzed and lyophilized (Zaneveld and Williams, 1970).

Enzyme Assays

Acrosin activity was measured using BAEE (Mann) as substrate according to the spectrophotometric procedure of Schwert and Takenaka (1955). Assays were performed in 0.05 M Tris–HCl buffer at pH 8.0 containing 0.05 M $CaCl_2$ after the third purification step. A unit of acrosin activity was arbitrarily defined as an absorbance change of 1 optical density (OD) U/min at a wavelength of 253 mμ and specific activity as acrosin units per milligram of protein (measured by absorption at 280 nm assuming 1 mg of protein/ml exhibits an OD at 280 of 1.0). Chymotrypsin assays were done using *N*-acetyl tyrosine ethyl ester (ATEE, Mann) as substrate and reading the rate of change as an optical density of 256 nm. Bovine pancreatic trypsin (TL OAB) and pancreatic trypsin inhibitor (PIC 8HD) were obtained from Worthington.

Chromatography

All chromatography was performed at 5C. Diethyl amino ethyl (DEAE) cellulose (Floc) was obtained from Whatman. The dry powder was suspended in 1 M sodium acetate, and the fine particles removed. The cellulose was washed with 0.5 M NaCl and 0.5 M HCl and finally resuspended in 0.1 M sodium acetate containing 0.003 M EDTA. Jacketed columns 2.5 cm in diameter and 36 cm long were prepared in 0.05 M phosphate buffer, pH = 7.0. The column was eluted stepwise by increasing the NaCl concentration.

Sephadex G-100 and G-75 (Pharmacia) were swollen and washed in 0.001 M HCl and columns 1.5 cm in diameter and 44 cm long were prepared. The protein was eluted from the column with 0.001 M HCl, pH 3.0. For the estimation of molecular weight known pure proteins were added to the same column according to the method of Whitaker (1963), and Andrews (1964). These reference proteins were ovalbumin, mol wt 43,000 for the monomeric form and 86,000 for the dimeric form (Pentex, 5 × crystallized, Code: 96010), bovine pancreatic trypsin, mol wt 23,300, lysozyme, mol wt 14,300 (Armour Lab.) and pancreatic trypsin inhibitor, mol wt 6,513. The void volume (v_0) was determined using dextran blue dye (Pharmacia). The mobility of the standards (the elution volume, v, of the protein divided by v_0) was plotted against the logarithm of the molecular weights and the molecular weight of acrosin was obtained through interpolation.

Polyacrylamide Gel Electrophoresis

Since acrosin was unstable after the third purification step the conventional disc electrophoresis techniques could not be employed. The preparations were therefore treated with 1.0% sodium dodecyl sulphate (SDS) to change the individual charge patterns of the proteins by the binding of SDS anions rendering all molecules negatively charged, the amount of charge depending on the size of the molecule. In addition, the preparations were treated with 1% mercaptoethanol to break all susceptible disulfide bonds. Treatment with SDS and mercaptoethanol converts most complex proteins to their subunits. The samples were then subjected to polyacrylamide disc electrophoresis in the presence of 0.1 M SDS according to the methods of Shapiro, Vinuela, and Maizel (1967), and Weber and Osborn (1969). This technique separates proteins only on the basis of their molecular weight.

Sodium dodecyl sulphate was obtained from Gallard Schlesinger Chemical Co. and 2-mercaptoethanol from Sigma Chemical Co. Polyacrylamide (10%) was prepared by dissolving 22.2 gm acrylamide (K and K Lab.), crystallized in benzene according to the method of Brewer and Ashworth (1969), and 0.6 gm methylene bisacrylamide (Eastman Organic) in 100 ml of water. Bromphenol blue (Mann) was used as tracking dye. The same technique for the preparation of the samples and disc electrophoresis was used as described by Weber and Osborn (1969) except that the final dialysis step was omitted. The gels were stained with Coomassie brilliant blue (Code: 11-152B, Colab Lab.). Ovalbumin, bovine pancreatic trypsin, lysozyme and pepsin were again used as reference proteins in addition to bovine serum albumin, mol wt 68,000 (Code: BV162, Pentex). The mobility of the proteins was calculated as:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before destaining}}{\text{distance of tracking dye migration}}$$

Using semilog paper the molecular weight was plotted against mobility. The molecular weight of acrosin was obtained by interpolation.

RESULTS

Purification of Acrosin. Step 1

The lyophilized acrosomal extracts were dissolved in 0.05 M phosphate buffer, pH 7.0 and centrifuged at 700 *g* for 15 min. The precipitate possessed only slight activity, indicating that acrosin is almost completely in soluble form. No chymotrypsin activity is present in the supernatant solution.

Step 2

The supernatant solution was cooled to 5C and added to a DEAE column. Acrosin does not adhere to the DEAE and was present in the first eluates (Fig. 1). Fractions 22–26 possessed the highest specific activity and were pooled separately from the fractions 27–31. The total acrosin activity almost doubles (Table 1).

After elution the enzyme is quite stable at 5C and the activity is not destroyed by freezing. This stability increases with increasing molarity of phosphate buffer.

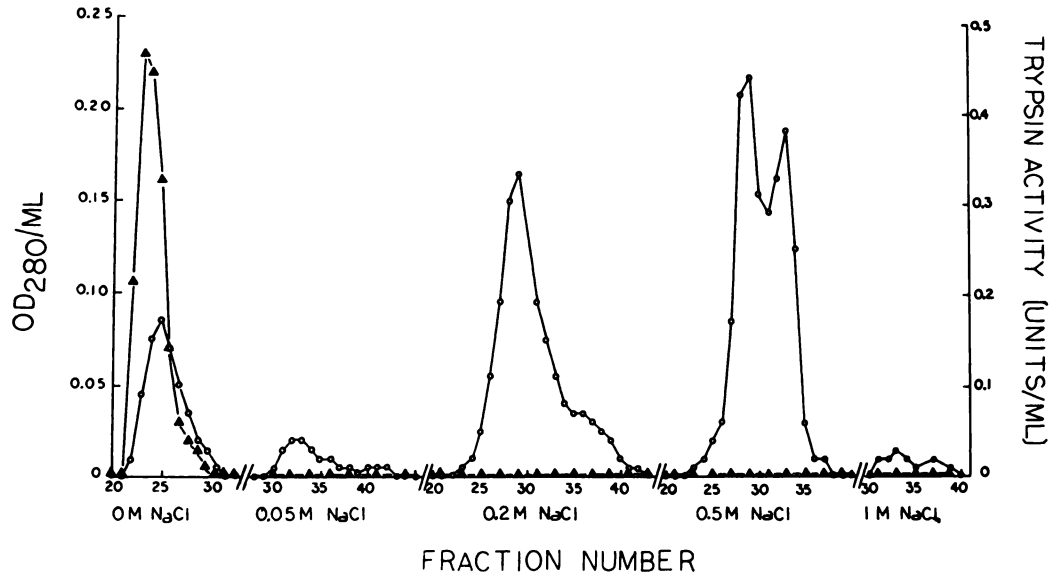

FIG. 1. Elution profile of centrifuged acrosomal extracts purified by DEAE-cellulose chromatography. The column was developed stepwise using increasing amounts of NaCl in 0.05 M phosphate buffer, pH 7.0. Each fraction contains 5 ml. ▲——▲ Acrosin activity, ○——○ Protein.

TABLE 1

PURIFICATION OF ACROSIN

	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Purification
Sperm acrosomal extracts	50.0	3.00	0.06	1
Centrifugation	15.2	2.70	0.18	3
DEAE chromatography	1.9	5.89	3.1	52
Sephadex G-100 chromatography	0.136	2.10	14.9	250

Step 3

To concentrate acrosin for further purification the phosphate buffer was removed by pooling fractions 21–26 and dialyzing against three 2,000 ml portions of 0.001 M HCl. The low pH prevents destruction during dialysis although loss of activity during freezing occurred after this purification step. The dialyzed preparation was lyophilized and was stored for several months without loss of activity.

Step 4

The lyophilized preparations were resuspended in 1–2 ml of 0.001 M HCl, added to a Sephadex G-75 or G-100 column and eluted with 0.001 M HCl (Fig. 2). The acrosin preparation obtained has a specific activity of 15 U/mg (Table 1), although a few contaminants are still present (Fig. 3). The amounts of these contaminants vary and are minor judging from their staining density after electrophoresis. Only the major band is seen consistently after Sephadex chromatography and consequently is acrosin.

Molecular Weight Determinations

Two Sephadex G-100 columns and one G-75 column were used to estimate the molecular weight. With the Sephadex G-75 only bovine pancreatic trypsin and pancreatic trypsin inhibitor were used as references but with the Sephadex G-100 columns all reference proteins except the pancreatic trypsin inhibitor were used. Through extrapolation (Fig. 4), molecular weights of

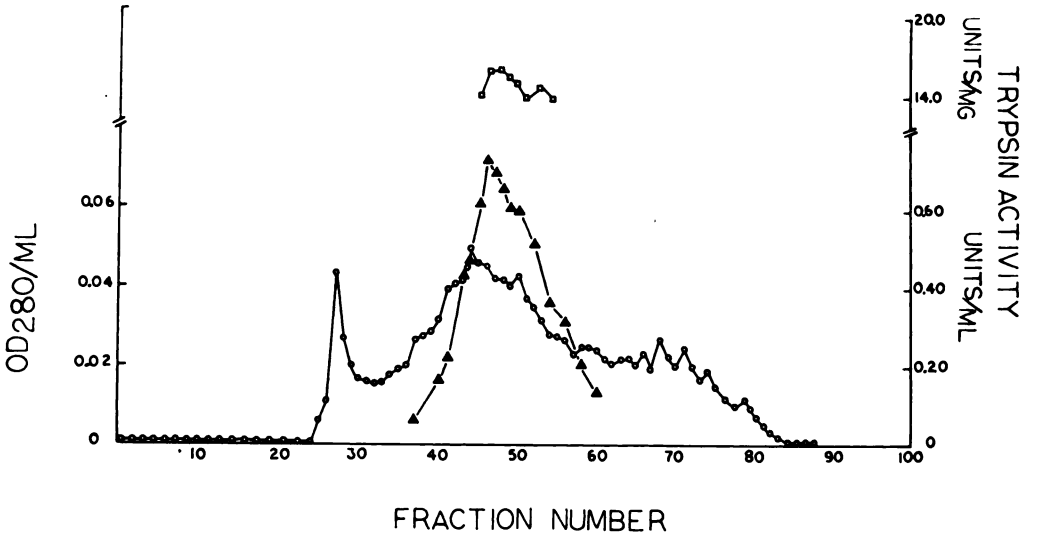

FIG. 2. Elution profile of DEAE-purified acrosomal extracts further isolated by Sephadex G-100 column chromatography. The column eluted with 0.001 M HCl. Each fraction contains 0.8 ml. O——O Protein, ▲——▲ Acrosin activity (U/ml), □——□ Acrosin activity (U/mg).

54,000; 55,000 and 56,000 (averaging 55,000) were obtained.

The protein band containing acrosin was readily identified in the SDS gels as the major band as it is the only one that consistently is present after Sephadex G-100 chromatography (Fig. 3). By comparison with references (Fig. 5) the molecular weight of acrosin was estimated as 27,000; 26,800

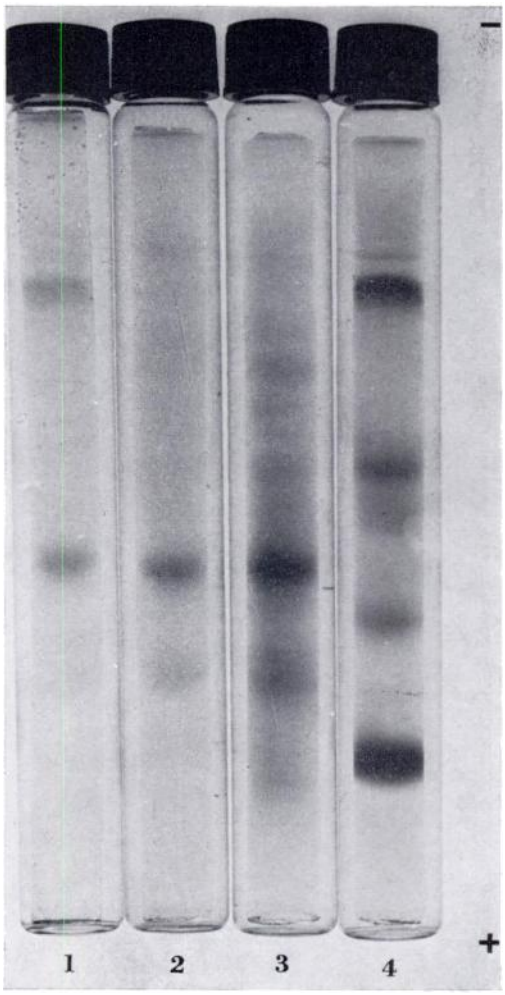

FIG. 3. Sodium dodecyl sulphate polyacrylamide electrophoresis of partially purified acrosomal extracts. 1 and 2 are after Sephadex G-100 chromatography (samples from 2 different columns)—Step 4. 3 is after DEAE-cellulose chromatography—Step 2. 4 is Standards (see text).

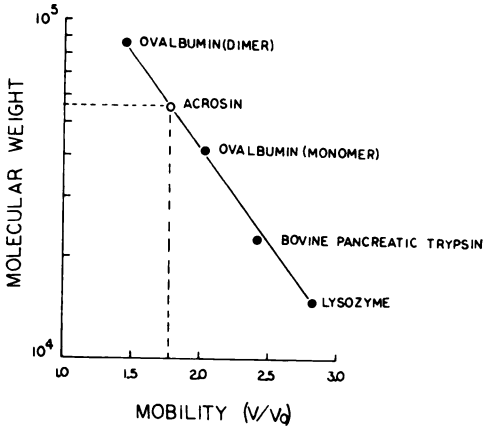

FIG. 4. Molecular weight estimation by Sephadex G-100 column chromatography.

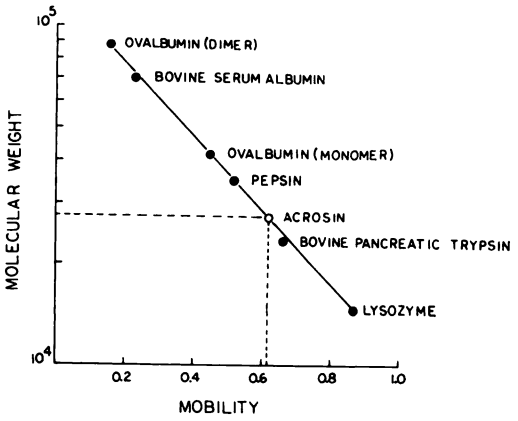

FIG. 5. Molecular weight estimation by sodium dodecyl sulphate polyacrylamide electrophoresis.

and 28,000 (averaging 27,300) for the acrosin bands obtained respectively after DEAE chromatography and two different passes through Sephadex G-100.

DISCUSSION

A 250-fold purification of acrosin was obtained and judging from the specific activity and SDS gel electrophoresis the enzyme preparation was almost pure. Dialysis at pH 3 was required in the third step since purified acrosin is unstable above pH 6 possibly due to autoproteolysis (Zaneveld, Polakoski, and Williams, 1971). This caused

many difficulties in further purification since all possible manipulations had to be performed in 0.001 N HCl thus eliminating ordinary polyacrylamide disc electrophoresis.

After DEAE chromatography, approximately a two-fold increase in total acrosin units was obtained. This was most likely caused by removal of a trypsin inhibitor similar to seminal plasma trypsin inhibitor (Zaneveld, Srivastava, and Williams, 1969). The presence of a small amount of trypsin inhibitor in the epididymal sperm acrosomal extracts is not surprising since the sperm are obtained by flushing the epididymis through the vas deferens which may contain some seminal fluid. It is also possible that epididymal fluid contains an inhibitor since Haendle (1968) reported the presence of small amounts of trypsin inhibitor in epididymal tissue.

The molecular weight of 55,000 for acrosin estimated by Sephadex chromatography should be compared with that obtained by Stambaugh and Buckley (1969) who reported a molecular weight of 59,000 for a acrosin–hyaluronidase complex using sucrose density gradient centrifugation. The evidence for a complex was the presence of hyaluronidase activity in the same part of the gradient as acrosin. They also suggested that acrosin and hyaluronidase together caused the dissolution of the zona pellucida. Since these data do not prove the existence of a complex and since testicular hyaluronidase does not effect the zona (Gould, 1970), their conclusions seem unjustified. Although Malmgren (1953) reported that the molecular weight of testicular hyaluronidase is 11,000, Borders and Raftery (1968) using highly purified preparations reported that the molecular weight is approximately 61,000. It is therefore not surprising that acrosin and the hyaluronidase were present in the same fraction after sucrose density gradient centrifugation since they have approximately the same molecular weight.

The molecular weight of acrosin as determined by SDS polyacrylamide gel electrophoresis is 27,300, about half of that obtained by Sephadex chromatography. Since the SDS and mercaptoethanol treatment reduce proteins to subunits and since acrosin is the only component that consistently is present after Sephadex chromatography, this suggests that acrosin exists as a dimer of molecular weight 55,000.

ACKNOWLEDGMENTS

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