PARTIAL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF RAT SPERM HYALURONIDASE

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ABSTRACT

Hyaluronidase (hyaluronoglucosaminidase; hyaluronate-4-glycanohydrolase, E.C. 3.2.1.35) from rat cauda epididymal sperm was extracted by the non-ionic detergent, Triton X-100. It had optimum activity at pH 4.0 and 37°C. The unstable nature of the enzyme during storage was observed.

Hyaluronidase from rat sperm was partially purified by using DEAE-cellulose chromatography, cellulose phosphate chromatography and Sepharose 4B chromatography. Cellulose phosphate column chromatography produced the highest purification and resulted in 232 turbidity reducing units per mg protein. Disc gel electrophoresis showed one major component and a minor band of faster mobility.

The K_m of hyaluronidase on hyaluronic acid was 0.40 mg per ml, while V_{max} was 15.9 mg hyaluronic acid digested per min per mg protein.

INTRODUCTION

Sperm hyaluronidase (hyaluronoglucosaminidase; hyaluronate-4-glycanohydrolase, E.C. 3.2.1.35) is an acrosomal enzyme released by the capacitated sperm for penetration of the cumulus oophorus, the mound of follicle cells (Fig. 1) which surrounds the ovum. It depolymerizes and hydrolyzes the hyaluronic acid matrix that cements the cells of the cumulus oophorus. The enzyme catalyzes the hydrolysis of the glucosaminidic bond between C_1 of the glucosamine and C_4 of the glucuronic acid moieties in hyaluronic acid.

Hyaluronidase of varying degrees of purity has been obtained from bull testes, and from sperm acrosomal extracts of bull, rabbit and ram. The enzyme from these sources has been studied with regards to stability, optimum pH, optimum temperature, cation requirements, K_m and V_{max}. No data are available concerning the characteristics of the enzyme from rat sperm, which in our experience is more available in the laboratory.

This study describes the partial purification and biochemical properties of rat sperm acrosomal hyaluronidase.

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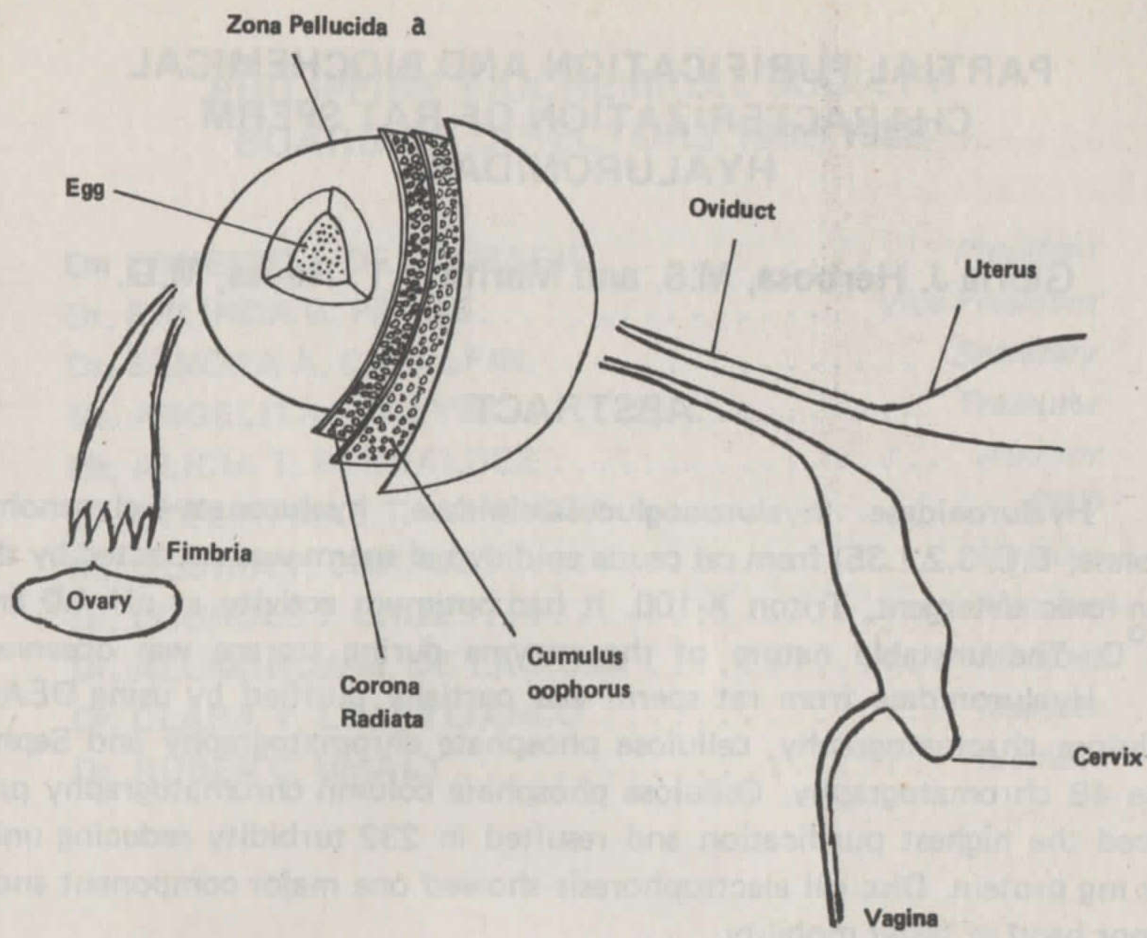

Fig. 1. Schematic representation of the ovum (1).

MATERIALS AND METHODS

Animal Sources. Male albino rats ranging from 100 to 300 days of age were obtained from several artificial breeding centers.

Reagents: Triton X-100, bovine testicular hyaluronidase (300 NF units per mg protein), hyaluronic acid (Grade 1, prepared from human umbilical cord), bovine plasma albumin (Fraction V powder), chondroitin sulfate A (Na salt, Type A, from whale cartilage), chondroitin sulfate B (Na salt, from porcine skin), Folin-Ciocalteu Phenol reagent and protein standards were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Heparin (1,000 USP units per ml, derived from porcine intestinal mucosa) was purchased from Abbott Laboratories, North Chicago, Illinois, U.S.A.

DEAE-cellulose and cellulose phosphate were obtained from Bio-Rad Laboratories, Richmond, California, U.S.A. Pre-swollen Sepharose 4B was supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

Polyacrylamide and N, N-methylene bisacrylamide used in the preparation of gels for disc electrophoresis were obtained from Eastman Kodak, Rochester, New York, U.S.A. N,N,N',N'-tetramethylethylenediamine (TEMED) was acquired from Canalco Industrial Corporation, Rockville, Maryland, U.S.A.

All other chemicals used were of the highest purity available.

Preparation of Acrosomal Extracts. Rat cauda epididymal sperm were collected and treated immediately by a modification of the methods of Cassilas and Chaipahyungan (1) and Polakoski et al. (2). The rat was first immobilized by cervical dislocation and the testicles dissected rapidly to remove the pair of cauda epididymis. The cauda epididymis were immediately placed on ice, lacerated with a sharp scalpel and flushed with cold 0.9% NaCl to solubilize epididymal spermatozoa. To remove epididymal plasma the sperms were repeatedly washed with cold 0.9% NaCl and centrifuged at 1,000 x g for 15 min. The final pellet was dispersed in 4 volumes of cold 0.9% NaCl and sonicated (Branson Cell Disruptor Model 185 D) in an ice bath using 15-sec bursts for 1 min. Sonicated sperm extracts from seven to ten male rats were pooled and incubated with 0.1% Triton X-100 in saline for 10 min based on the method of Hartree and Srivastava (3). The resulting acrosomeless sperms were removed by centrifugation at 1,000 x g (SS-34 rotor of RC2-B Refrigerated Sorvall Centrifuge) for 15 min. The supernatant solution was labelled the acrosomal extract.

Hyaluronidase Activity Assay. Hyaluronidase activity was determined by a modification of the methods of Toksdorf et al. (4) and Kas and Seastone (5) as described previously. Hyaluronic acid concentration was measured by its ability to turn turbid an acid albumin solution. The assays were performed at 37°C.

One turbidity reducing unit (the unit of hyaluronidase activity) was defined as that amount of enzyme which decreases the turbidity producing capacity of 0.2 mg hyaluronic acid (HA) to that of 0.1 mg HA in 30 min at 37°C.

Turbidity reducing unit (TRU) = mg HA digested x (3/0.1) in 10 min. Specific activity was expressed as units per milligram of protein.

Protein Determination. The amount of protein was estimated by the method of Lowry, et al. (6) using bovine serum albumin as standard.

Optimum pH. The pH optimum of rat sperm acromosal hyaluronidase was determined using 0.1 M phosphoric acid containing 0.15 M NaCl. The pH of the enzyme and the substrate solutions were adjusted using 0.1 M NaOH and 0.1 M HCl.

Optimum Temperature. The optimum temperature of rat sperm acrosomal hyaluronidase was determined by incubating the enzyme and substrate in a water bath for 10 min. at various temperatures from 0-60°C.

DEAE-Cellulose Ion Exchange Chromatography (7, 8). Fifteen ml of acrosomal extract (13.0 mg protein) was applied to a DEAE-cellulose column (2.1 x 58 cm) which has previously been equilibrated at 0-4°C with 0.05 M sodium phosphate buffer pH 7.0. The chromatogram was developed with a linear gradient of 0 to 0.5 M NaCl at a flow rate of approximately 2.0 ml per min.

Cellulose Phosphate Column Chromatography (8, 9). Twenty ml

acrosomal extract (31 mg protein) was applied to a cellulose phosphate column (2.1 x 58 cm) previously equilibrated at 0-4°C with 0.05 M sodium phosphate buffer, pH 7.0. The column was eluted with a linear gradient of 0.5 M NaCl in 0.05 M sodium phosphate buffer pH 7.0 at a flow rate of approximately 1.0 ml per min.

Determination of K_m and V_{max}. Fifty microliters of the pooled active cellulose phosphate fractions was pipetted into a series of tubes containing various concentrations of the hyaluronic acid substrate ranging from 0 to 0.4 mg per ml to make a total volume of 100 μl of reaction mixture. Controls were made by omitting the substrate. The amount of hyaluronic acid digested by the sample in 10 min was determined as previously described. A Lineweaver-Burke plot was constructed with the reciprocal of the substrate concentration as the abscissa and the reciprocal of the initial reaction velocity (expressed as mg hyaluronic acid digested per min per mg protein) as the ordinate.

Sepharose 4B Column Chromatography (9). A Sepharose 4B column 1.3 x 50 cm long was equilibrated with 0.1 M monosodium phosphate buffer pH 5.3, containing 0.15 M NaCl. Chromatography was performed at 0 to 4°C at a flow rate of 2 ml per 10 min. The void volume was determined using Blue Dextran. Two mg each of the following standards was applied: hexokinase, catalase and blue dextran. Around 1.5 ml of acrosomal extract (5.9 mg protein) previously concentrated in polyethylene glycol was applied to the column.

Polyacrylamide Disc Gel Electrophoresis. Active fractions from the different chromatography runs were applied on polyacrylamide gels. Electrophoresis was carried out at pH 9.5 by the method described in the Canalco Research Disc Electrophoresis Manual (10) on 7.5% acrylamide gel with bromphenol blue as tracking dye. A current density of 4 mA per gel was applied until the tracking dye was 1 cm from the bottom of the gel tube. The gels were stained with 0.1% Coomassie brilliant blue and destained with 7% acetic acid.

The relative mobility of each protein band was computed as follows:

$$\text{Relative Mobility} = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

RESULTS

Extraction of Hyaluronidase with Detergent. Table 1 compares hyaluronidase activities of sonicated sperm (NSS) extract and the same extract after treatment with detergent (Triton X-100). Enzyme specific activity increased by about 80% (5-fold) after detergent extraction.

Table 1. **Detergent Extraction of Rat Sperm Hyaluronidase**

	NSS Extract	Detergent Extract
Total Volume (ml)	20.0	40.0
Protein Concentration (mg/ml)	0.400	0.160
Total Protein (mg)	7.92	6.20
Enzyme Activity (TRU/ml)	5.30	10.5
Specific Activity (TRU/mg protein)	13.3	65.6
Total Activity (TRU)	105	407
Relative Activity	1	5

Table 2. **Stability of Hyaluronidase**

Condition	% Loss of Enzyme Activity
Temperature	
0 – 10°C, 2 hrs.	0
60°C, 2 hrs.	100
freezing and thawing	100
refrigeration, 0-4°C, 24 hrs.	50
Dialysis against distilled H_2O	0
Lyophilization	87

Optimum pH. Rat sperm acrosomal hyaluronidase had optimum activity at pH 4.0 (Fig. 2). There was activity within the pH range of 3.0 to 7.0. The enzyme had less than 30% activity at pH 6.0.

Optimum Temperature. Rat sperm acrosomal hyaluronidase had an optimum temperature of 37°C (Fig. 3). No activity was detected at 60°C.

DEAE-Cellulose Column Chromatography. The elution profile of protein and hyaluronidase activity in rat acrosomal extract on DEAE-cellulose column is shown in Fig. 4.

Five major protein peaks were obtained from the DEAE-cellulose column. Significant hyaluronidase activty was detected mostly in the second and third protein peaks. The fraction with the highest specific activity was eluted at 0.11 M NaCl. Several runs showed the same results.

Cellulose Phosphate Column Chromatography. The elution profile of protein and hyaluronidase activity of rat acrosomal extract in the cellulose phosphate column is shown in Fig. 5.

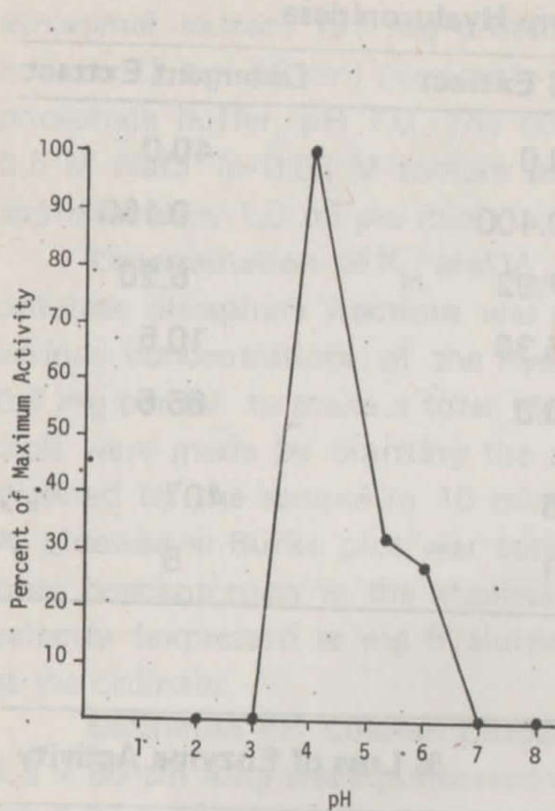

Fig. 2. The pH optimum of rat acrosomal hyaluronidase.

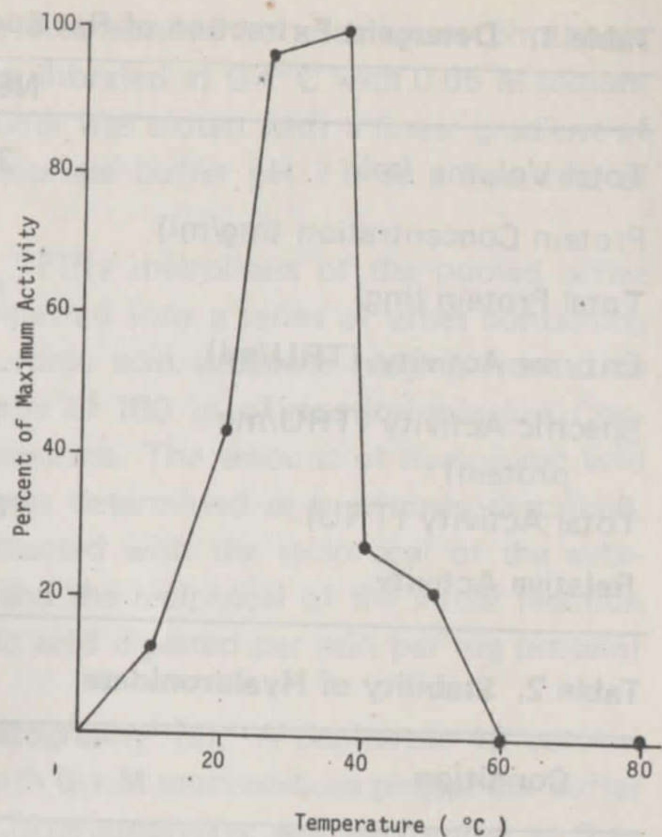

Fig. 3. The effect of temperature on rat acrosomal hyaluronidase activity.

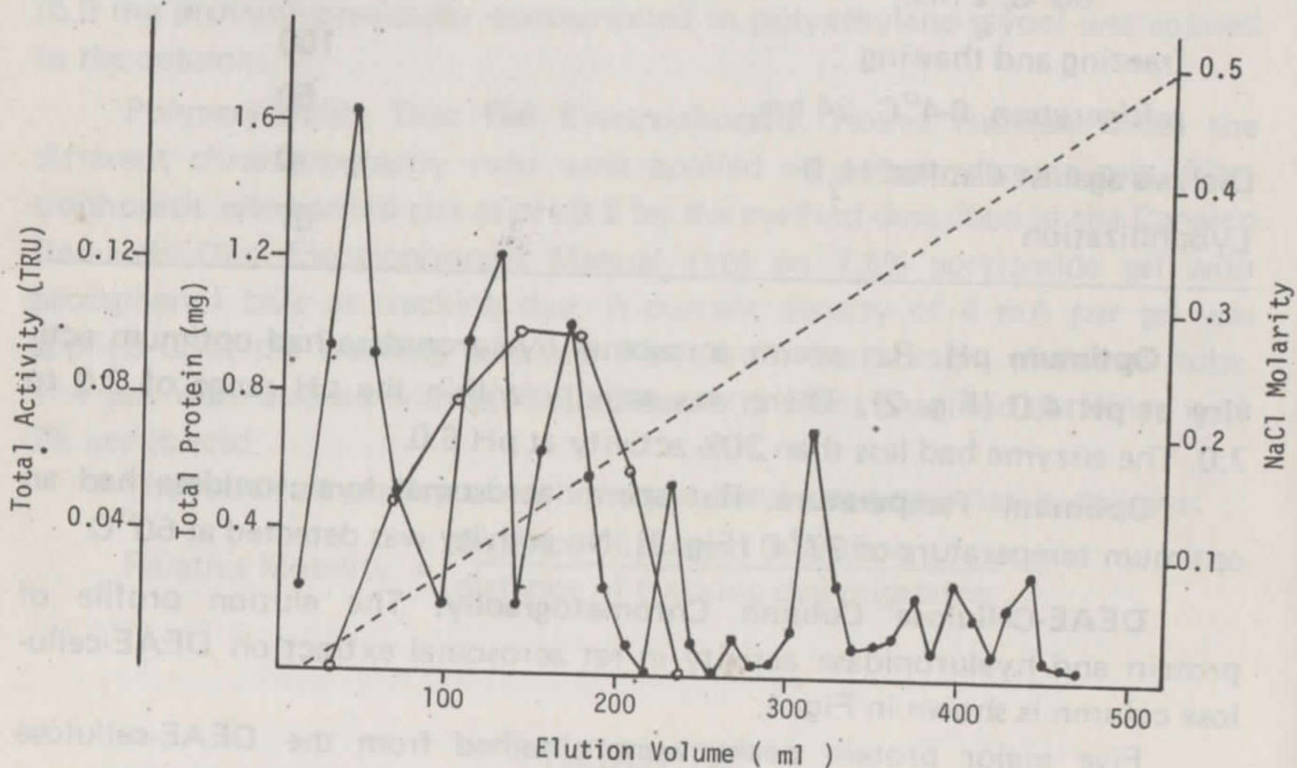

Fig. 4. DEAE-cellulose column chromatography of acrosomal extract from rat cauda epididymal sperm. ● — ● protein concentration; ○ — ○ hyaluronidase activity. Fifteen ml of acrosomal extract (13.0 mg protein) in 0.05 M sodium phosphate buffer, pH 7.0 was applied to the column and eluted with the same buffer using a linear salt gradient.

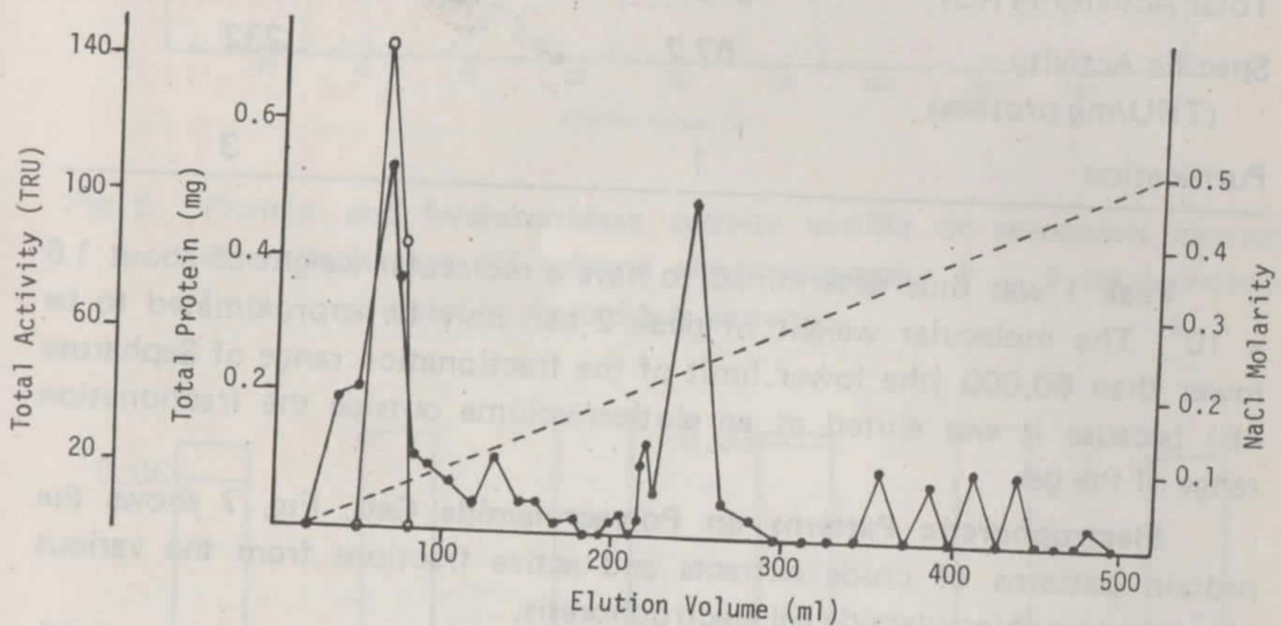

Fig. 5. Cellulose phosphate column chromatography of acrosomal extract from rat cauda epididymal sperm ● — ● total protein concentration (mg); ○ — ○ hyaluronidase activity. The acrosomal extract (31.0 mg protein) in 0.05 M sodium phosphate buffer, pH 7.0 was applied to the column and eluted with the same buffer using a linear salt gradient.

Rat acrosomal hyaluronidase appeared in the first protein fraction. A 3-fold increase in specific activity (Table 3) was detected at 0.05 M NaCl. The resolution of proteins in the cellulose phosphate column was better than in the DEAE-cellulose column.

Sepharose 4B Column Chromatography. The elution profile on Sepharose 4B is shown in Fig. 6.

Two peaks with hyaluronidase activity were obtained. One was detected at an elution volume of 31.5 ml where there was undetectable protein concentration. The other slightly more active peak was eluted in the elution volume of bromphenol blue. The major protein peak was eluted in the same volume.

Table 3. Partial purification of rat acrosomal hyaluronidase by cellulose phosphate column chromatography.

	Sperm Acrosomal Extracts	Cellulose Phosphate Column Chromatography
Total Protein (mg)	31.0	0.91
Total Activity (TRU)	210	212
Specific Activity (TRU/mg protein)	67.7	232
Purification	1	3

Peak 1 was thus determined to have a molecular weight of about 1.6 x 10^6. The molecular weight of peak 2 can only be approximated to be lower than 60,000 (the lower limit of the fractionation range of Sepharose 4B) because it was eluted at an elution volume outside the fractionation range of the gel.

Electrophoretic Patterns on Polyacrylamide Gels. Fig. 7 shows the protein patterns of crude extracts and active fractions from the various columns on polyacrylamide gel electrophoresis.

Two bands: a slower moving band with mobility varying from 0.57-0.76 and a faster one with mobility ranging from 0.93-1.0 were consistently present in gel patterns C, D and E (cellulose phosphate active fractions, Sepharose 4B active peaks 1 and 2, respectively). These bands are also present in gel pattern F which is from type 1 bovine testicular hyaluronidase (Sigma (H)) with mobilities of 0.65 and 0.96, respectively.

Gel pattern A (crude acrosomal extract) exhibited 4 bands including bands with mobilities of 0.64 and 0.92 which are consistent with those found in the gel patterns of the partially purified fractions.

Gel pattern B (active fraction from DEAE-cellulose column) had other protein bands aside from two with mobilities 0.67 and 0.96.

Determination of K_m and V_{max}. Kinetic studies performed at 37°C using active cellulose phosphate fractions with hyaluronic acid as substrate gave an apparent K_m value of 0.40 mg per ml and a V_{max} of 15.9 mg hyaluronic acid digested per min per mg protein as obtained from extrapolation of the Lineweaver-Burk plot as shown in Fig. 8. The Lineweaver-Burk plot is linear below 0.13 mg per ml but shows inhibition at higher substrate concentrations.

DISCUSSION

Sperm hyaluronidase has been shown to appear first in the testes during spermatogenesis (11, 12). However, spermatozoa obtain their

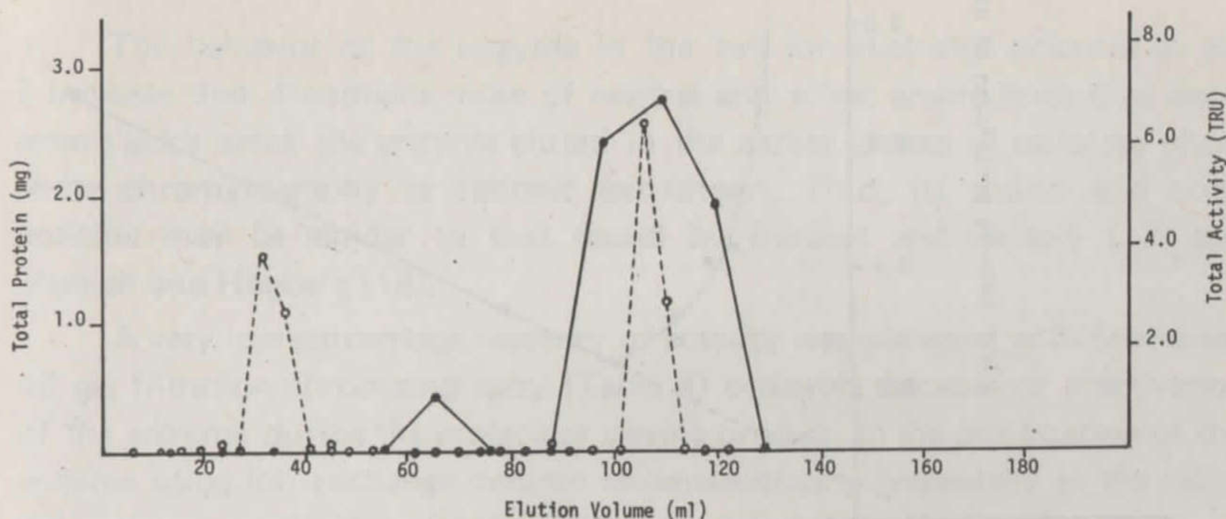

Fig. 6. Protein and hyaluronidase activity profile of acrosomal extract after Sepharose 4B column chromatography ● — ● total protein (mg); ○ — ○ total hyaluronidase activity.

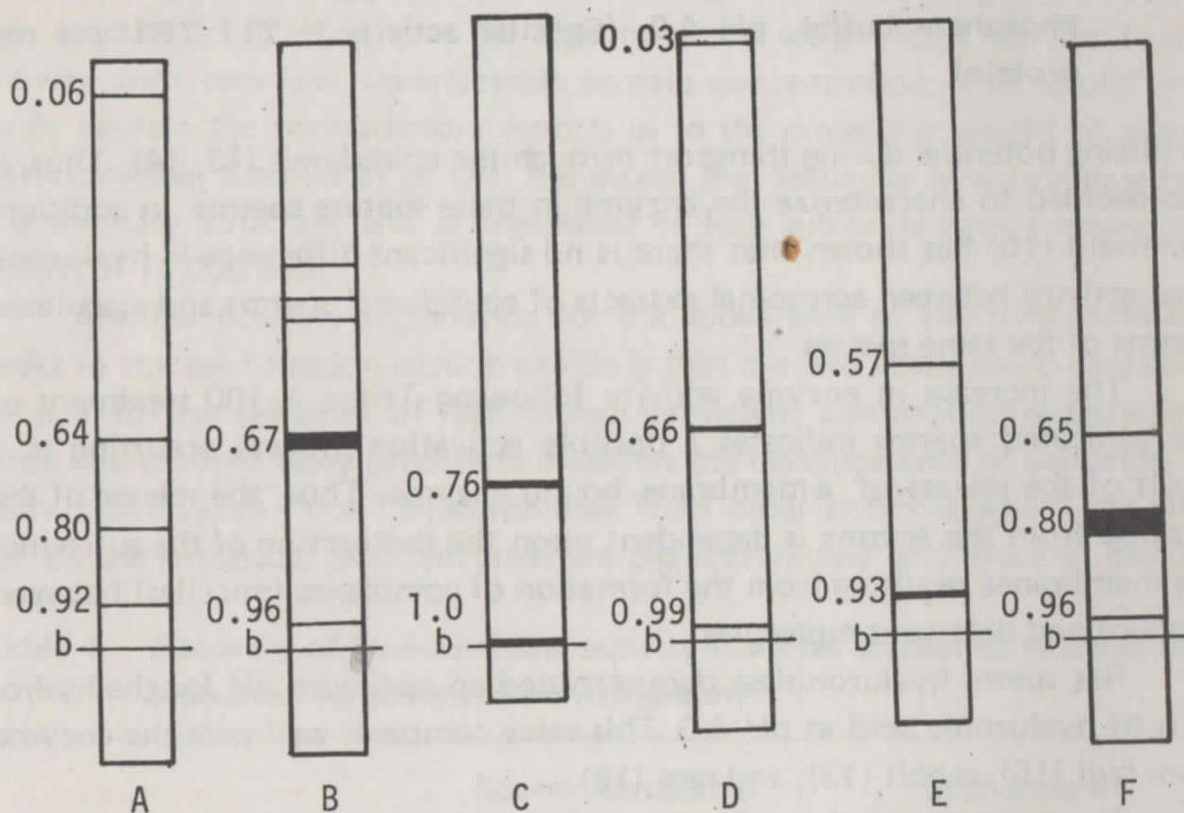

Fig. 7. Polyacrylamide disc gel electrophoretic patterns of various fractions. One hundred µl of protein solution (50 µg protein) was applied on a 7-1/2% gel. Electrophoresis was carried out at pH 9.0, 4 mA per gel tube for 2 hours. A — acrosomal extract; B — DEAE-cellulose active fraction; C — Cellulose phosphate active fraction; D — Sepharose 4B active peak 1; E — Sepharose 4B active peak 2; F — Type 1 bovine testicular hyaluronidase (Sigma). The number beside each band refers to relative mobility. b refers to position of the bromphenol blue marker.

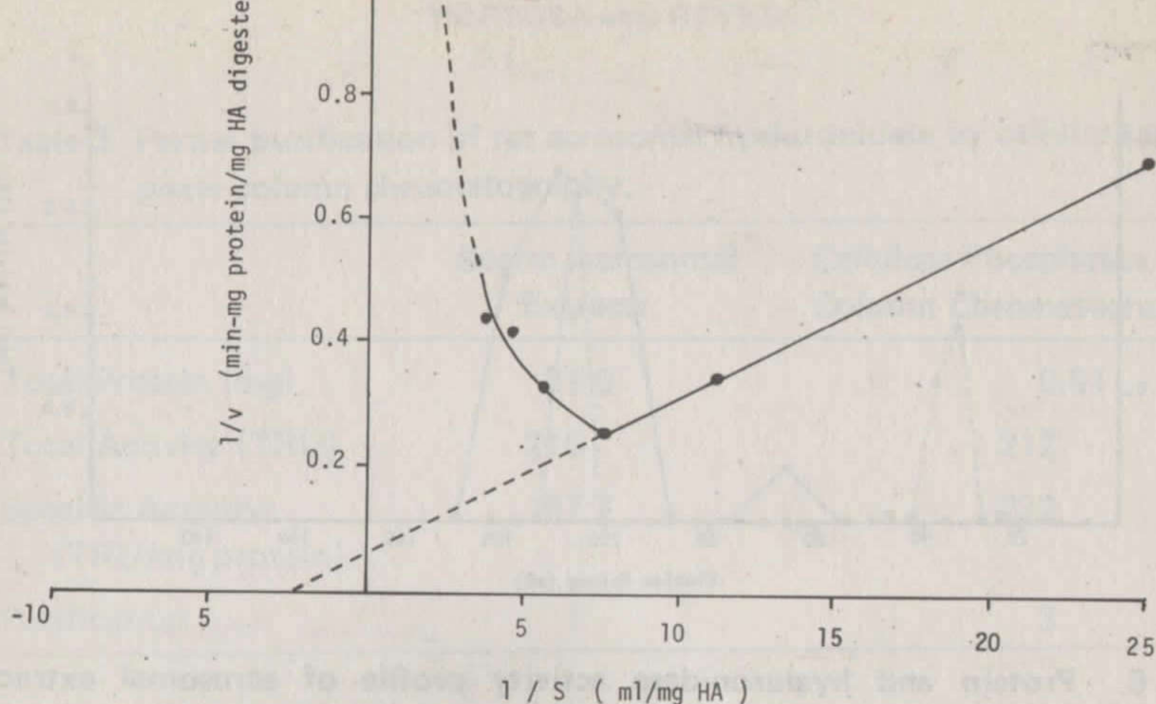

Fig. 8. Lineweaver-Burk plot for the determination of K_m and V_{max}. The action of rat sperm hyaluronidase on hyaluronic acid was assayed using partially purified enzyme at 37°C in NaCl and 0.1 M sodium phosphate buffer, pH 4.0. (Specific activity = 711 TRU per mg protein).

fertilizing potential during transport through the epididymis (13, 14). Thus, it was decided to characterize the enzyme in these mature sperms. In addition, Zaneweld (15) has shown that there is no significant difference in hyaluronidase activity between acrosomal extracts of epididymal sperms and ejaculated sperms of the same species.

The increase in enzyme activity following Triton X-100 treatment of the sonicated sperms indicates a possible activation process occurring as a result of the release of a membrane-bound enzyme. Thus, the release of the enzyme from the sperms is dependent upon the destruction of the surrounding membranes resulting from the formation of complexes (micelles) between proteins and detergent molecules.

Rat sperm hyaluronidase demonstrated an optimum pH for the hydrolysis of hyaluronic acid at pH 4.0. This value compares well with the enzyme from bull (15), rabbit (13), and ram (16).

The temperature for optimum activity of the enzyme was determined to be 37°C. Like its bull counterpart (15), rat sperm hyaluronidase lost most of its activity at 55°C and all of its activity at 60°C. Rat sperm hyaluronidase activity was lost during freezing and thawing, while Zaneweld (15) reported no significant alteration of bull sperm hyaluronidase activity by the same procedure. This indicates more lability of the rat sperm hyaluronidase. Indeed, storage and lyophilization resulted in a marked decrease in rat sperm hyaluronidase activity of the acrosomal extracts (Table 2). The purification techniques employed (14, 15, 16) were thus one-step processes, considering the apparent instability of the enzyme upon long standing.

The behavior of the enzyme in the two ion exchange columns at pH 7 indicate that it contains more of neutral and acidic amino acids than basic amino acids since the enzyme eluted in the earlier phases of cellulose phosphate chromatography (a cationic exchanger). Thus, its amino acid composition may be similar to that found by Borders and Raftery (17) and Bronesh and Hogberg (18).

A very low percentage recovery of activity was obtained with Sepharose 4B gel filtration chromatography (Table 4) probably because of inactivation of the enzyme during the molecular sieving process. In the purification of the enzyme using ion exchange column chromatography (especially in the cationic exchanger, cellulose phosphate) it was noted that the enzyme eluted out first before the other proteins so that decrease in activity due to binding with the exchangers was minimal.

The 2 peaks of hyaluronidase activity found, having molecular weights of 1.5×10^6 and 60,000 daltons, may be explained by the association of enzyme units into an aggregate structure of high molecular weight. This could also account for the fact that the high molecular weight form had significant activity with very low, undetectable protein concentration. This would probably explain the contradictory reports as to the molecular weight of sperm hyaluronidase. Khorlin et al. (19) has shown that testicular hyaluronidase has a quaternary structure and is composed of four sub-units with a molecular weight of 14,000 each.

Another possible explanation for the appearance of two hyaluronidase peaks in the gel filtration elution profile is that the two peaks could probably be due to the presence of high molecular weight contaminating enzymes. Since the enzyme assay procedure measures the disappearance of substrate, it cannot distinguish "true" hyaluronidase from other enzymes which can also act on the substrate. Such enzymes are β-glucuronidase and N-acetyl glucos-

Table 4. Recovery of hyaluronidase activity from rat acrosomal extracts by Sepharose 4b column chromatography.

	Sperm Acrosomal Extract	Sepharose 4B Chromatography	
		Peak 1	Peak 2
Total protein (mg)	5.90	2.76	--[a]
Total activity (TRU)	2960	93.6	65.4
Specific activity (TRU/mg protein)	502	33.9	--
Percent recovery (%)		3.16	2.2

[a] *undetectable level.*

aminidase. Although conditions for their optimal activities may vary somewhat from those of hyaluronidase, future work on hyaluronidase (especially with regard to its purification) should include assays of β-glucuronidase and N-acetyl glucosaminidase. They have also been found to be present in sperm acrosomes although their functions are not known (20). Only hyaluronidase is known to be involved in the hydrolysis of the hyaluronic acid matrix of the cumulus oophorus.

The electrophoretic patterns of the purified fractions on polyacrylamide gels lend support to the observations in the extraction and purification procedures used. The two protein bands with average relative mobilities of 0.63 and 0.95 found in the electrophoretic patterns of the standard bovine testicular hyaluronidase (Type 1, Sigma) and consistently found in the gel patterns of all the active fractions and extracts point to the possibility that these are active hyaluronidase bands.

The rather heterogeneous gel pattern of the DEAE-cellulose active fraction is consistent with the observation from the DEAE-cellulose elution profile where hyaluronidase activity was detected in four peaks. The gel pattern showed again the presence of the two bands with relative mobilities of 0.67 and 0.96. It means that these two moieties were eluted from the anion exchanger at different ionic strengths encompassing four protein peaks. This could probably be explained by various stages of aggregation such that these two moieties joined other proteins of similar charges during binding to the exchanger. Thus, the three additional protein bands of low mobilities may represent various stages of aggregation with an increasing negative charge.

The electrophoretic pattern of the cellulose phosphate active fraction showed solely the two bands with practically the same mobilities as in the gel pattern for standard hyaluronidase. These protein moieties have the same ionic charge since they were eluted out at the same unit strength but have different molecular weights since their relative mobilities vary. It can be concluded therefore that a relatively pure sample was obtained. Probably the relative success in purification may be explained by the fact that no binding with the cation exchanger occurred. Hence, there was no chance for the enzyme to elute at other ionic strengths as a result of aggregation with other proteins.

Based on the electrophoretic patterns of the different purified fractions on the polyacrylamide gels and the degree of purification obtained for the different methods used, the cellulose phosphate fraction showed the highest specific activity with minimum number of contaminants. Thus it was used to determine kinetic constants of the enzyme. The K_m value obtained for partially purified rat sperm hyaluronidase was 0.40 mg hyaluronic acid per ml. This suggests high affinity of the rat enzyme for the substrate. Future work on the enzyme could be facilitated using the rat as the source because smaller amounts of the expensive substrate can be used.

The Lineweaver-Burk plot (Fig. 8) demonstrates an example of substrate inhibition at high concentrations of substrate. Physiologically, the inhibition of hyaluronidase by the substrate may be of great significance in the matter of regulating sperm penetration through the cumulus oophorus. It can be recalled that the sperm merely lyzes a path through the cumulus oophorus and the retention of the cumulus oophorus around the egg during fertilization is necessary for directing the sperm toward the zona. Thus, the substrate itself, the hyaluronic acid matrix holding the cumulus oophorus cells together, prevents its own further digestion.

Inhibition may be caused by dead-end combination of the substrate with the form of the enzyme it is not supposed to bind with. Further studies on the kinetics of this observed inhibition is needed for the elucidation of the mechanism of the catalytic activity as well as for understanding of the inhibitory action of the substrate.

CONCLUSION

The partial purification and characterization of rat sperm hyaluronidase, the first enzyme released by the sperm in penetrating the ovum prior to fertilization, was achieved. Enzyme extraction with the detergent, Triton X-100, DEAE-cellulose chromatography, cellulose phosphate chromatography and Sepharose 4B chromatography were used. A 3-fold purification was obtained with cellulose phosphate chromatography with highest recovery of enzyme activity. A low recovery of enzyme activity was noted with gel filtration chromatography. Inactivation of the enzyme during the molecular sieving process is proposed as possible explanation for such observations.

Rat sperm possesses an enzyme which digests hyaluronic acid. Its optimum pH for hydrolysis of the glycosaminoglycan is at pH 4.0 with a range of activity from pH 3.0 to 7.0. The optimum temperature for enzymatic activity was 37°C. Storage and lyophilization caused a marked decrease in activity. Freezing and thawing also caused inactivation of the enzyme.

The K_m for rat sperm hyaluronidase was found to be 0.40 mg per ml with V_{max} of 15.9 mg hyaluronic acid digested per min per mg protein. Inhibition at high concentrations of the substrate was observed.

The molecular weight and sub-unit structure of the enzyme were not fully studied as a result of its rather unstable nature. However, preliminary studies show that the enzyme probably consists of several units aggregated into a high molecular weight form capable of dissociating into smaller units which retain a significant degree of enzymatic activity.

ACKNOWLEDGMENT

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