Purification and Properties of Hyaluronidase from Bull Sperm*

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SUMMARY

Hyaluronidase from bull sperm was fractionated by ammonium sulfate and further purified by DEAE-cellulose and Sephadex chromatography. The highly purified hyaluronidase preparation showed 2,370 units per mg of protein (68,730 N.F. units per mg of protein), i.e. 182-fold purification. Disc gel electrophoresis showed one major component. The molecular weight of bull sperm hyaluronidase was 62,000 by sodium dodecyl sulfate gel electrophoresis.

Hyaluronidase from bull sperm has optimum activity at pH 3.8 and an absolute requirement for cations. K+ and Na+ have a greater effect than Ca2+, Mg2+, and Mn2+, whereas Co2+, Cu2+, and Zn2+ do not affect the enzyme activity. Purified preparations are less stable than crude extracts stored frozen at -15°C. KM of hyaluronidase with hyaluronic acid as substrate is 3.7 mg per ml and Vmax is 2.4 μmol per min by Hofstee plot.

Bovine testicular hyaluronidase has been purified to varying degrees of homogeneity and activity (1-4). Testicular hyaluronidase is localized exclusively in acrosomes of developing spermatids and spermatozoa as shown by immunofluorescent staining of the intact cells (5) and by separation of sperm heads before disrupting acrosomes (6). Masaki and Hartree (7) observed that upon storage of normal bull semen hyaluronidase diffused from spermatozoa to the seminal plasma. They stated that the enzyme is localized in spermatozoa as vasectomy led to disappearance of hyaluronidase from the semen (8, 9). Azospermic human semen is also devoid of the enzyme (10). Recently, Zaneveld et al. (11) reported a partial purification and properties of acrosomal hyaluronidase from frozen stored spermatozoa of the bull and showed that acrosomal hyaluronidase was identical with testicular hyaluronidase by immunodiffusion studies. In view of the fact that most of hyaluronidase would have leaked into the seminal plasma on storage, we compared the activity of the partially purified acrosomal hyaluronidase (11) to the activity of the enzyme present in the seminal plasma. The activity of hyaluronidase in seminal plasma was extremely high. For these reasons, we chose bull seminal plasma as the starting material for obtaining sperm-acrosomal hyaluronidase of greater purity and higher specific activity than hitherto reported. Since the initial source of hyaluronidase in seminal plasma was sperm acrosome the enzyme will be referred to as sperm hyaluronidase for comparison with the enzyme purified by other workers.

EXPERIMENTAL PROCEDURE

Materials—Bull semen was the gift from several artificial breeding centers. Different batches of pooled bull semen received during the course of 6 months were stored at -20°C until used. Hyaluronic acid (Grade III-P, Lot 51C-2270), DEAE-cellulose (medium mesh, Lot 30C-2380), and p-dimethylanilinobenzaldehyde were purchased from Sigma Chemical Co. Sephadex G-75 and G 100 both in bead form (40 to 120 μ) were obtained from Pharmacia Fine Chemicals. National Formulary reference standard of hyaluronidase was obtained from the American Pharmaceutical Association. All other chemicals were of the highest purity available. Plastic vessels were used to protect purified hyaluronidase from inactivation by contact with glass (12).

Enzyme Assays—Hyaluronidase activity was determined colorimetrically using hyaluronic acid as substrate in 0.02 M sodium acetate buffer (pH 3.8) containing 0.4 M NaCl (13). β-N-Acetylglucosaminidase activity was determined by the method of Pugh et al. (14) using p-nitrophenyl-N-acetylβ-D-glucosaminide (Calbiochem) as the substrate. β-Glucuronidase activity was determined using phenolphthalein glucuronide by the method of Fishman et al. (15).

Protein was estimated by the method of Lowrey et al. (16) using bovine serum albumin as standard. Biuret reagent was used for estimating high concentrations of protein according to the procedure of Gornall et al. (17).

One colorimetric unit is that amount of enzyme which releases 1 μmol of N-acetylglucosamine in 1 min at 37°C. The National Formulary standard hyaluronidase has a potency of 8.8 N.F. units per mg of solids or 230 N.F. units per mg of protein. One milligram of protein of the National Formulary standard hyaluronidase was equivalent to 7.93 units by colorimetric assay, i.e. 1 colorimetric unit = 20 N.F. units.

Purification of Bull Sperm Hyaluronidase—Thawed bull semen was first centrifuged at 5,000 × g for 10 min to remove spermatozoa and the seminal plasma supernatant was subsequently centrifuged at 105,000 × g for 4 hours in order to remove the pellet containing the decapacitation factor (18). The supernatant from the high speed centrifugation was dialyzed for 48 hours at 4°C against 0.01 M sodium phosphate buffer (pH 7.1) changing the buffer at 4, 12, and 24 hours. The nondialyzable solution was brought to 30% saturation by adding solid (NH4)2SO4 at 4°C and allowed to stand overnight. After removal of the precipitate, the supernatant was brought to 65% saturation with (NH4)2SO4, the mixture allowed to stand overnight at 4°C and centrifuged at 10,000 × g for 30 min. The precipitate was dissolved in distilled water and dialyzed against distilled water. The insoluble precipitate that formed during the first 10 hours of dialysis was removed by centrifugation at 15,000 × g for 20 min and the resultant supernatant was lyophilized.

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The lyophilized material (101 mg of protein) was dissolved in 0.02 M potassium phosphate buffer, pH 6.0, containing 0.15 M NaCl and applied to a column of Sephadex G-100 (2.5 × 93 cm) at 4° equilibrated with the same buffer (Column I). The column was washed with 250 ml of the buffer at the rate of 94 ml per hour. The hyaluronidase-active fractions eluting in the first protein peak were pooled and concentrated to 20 ml. The concentrated solution (25 mg of protein) was applied to a DEAE-cellulose column (2.5 × 36 cm) at 4° equilibrated with 0.005 M potassium phosphate buffer, pH 7.1 (Column II). The column was washed with 300 ml of the buffer and developed with a stepwise gradient of 0.05, 0.1, 0.15, 0.22 M NaCl at the rate of 27 ml per hour. The hyaluronidase-active fractions eluting in the first protein peak with the buffer alone were pooled and concentrated by ultrafiltration (Amicon Diaflo, PM-10 filter) with several additions of 0.15 M NaCl solution at 4°. The purified hyaluronidase obtained from above (3.3 mg) was applied to a Sephadex G-75 column (2.5 × 88 cm) which had been equilibrated with 0.15 M NaCl at 4° (Column III). The flow rate was adjusted to 10 ml per hour and 2.5-ml fractions were collected. The tubes containing hyaluronidase of constant specific activity were pooled (155 μg). The scheme of purification is outlined in Fig. 1.

**Polyacrylamide Gel Electrophoresis—**Disc gel electrophoresis was carried out at pH 2.3, 4.3, and 5.3 by the method of Brewer and Ashworth (19) on 5, 7.5, and 10% acrylamide gel with methyl green as a tracking dye. A current density of 1.5 ma per gel was applied for 3 hours. The gels were stained with 1%; p-mercaptoethanol to break all susceptible disulfide bonds. The samples were then subjected to polyacrylamide gel electrophoresis in the presence of 0.1 M sodium dodecyl sulfate (pH 6.8; 10% gel). The mobility of unknown protein and standard was plotted against molecular weight on a semilog paper. Ovalbumin, bovine pancreatic trypsin, lysozyme, pepsin, and bovine serum albumin were used as reference standards.

**RESULTS**

Hyaluronidase activity was negligible in the sedimented sperm (3,000 × g) and in the pellet of the second centrifugation (105,000 × g) compared to the seminal plasma. From 159 ml of seminal plasma (76 mg per ml of protein), 143 ml (4.5 mg per ml of protein) of supernatant solution were obtained after centrifugation. The protein precipitated by 65% (NH₄)₂SO₄ contained hyaluronidase. This protein was dissolved in distilled water and dialyzed against the same buffer. By removing the precipitate formed during dialysis, 66% of the contaminating proteins with little hyaluronidase activity were removed and 75% of the total hyaluronidase was recovered in the clear non-dialyzable fraction. This step was essential for obtaining highly purified enzyme.

The quantitative data on purification is summarized in Table I. Using Column I the low molecular weight contaminants in the second peak were separated from hyaluronidase. NaCl in the eluting buffer stabilized the enzyme. Four components were separated by Column II. The first peak eluted by buffer alone showed high hyaluronidase activity but the other three protein peaks had none. The fractions containing hyaluronidase (Column III) were pooled, concentrated by Dialflo, and applied to Column IV. The tubes having constant specific activity (Column IV, Fig. 2) were pooled, concentrated by Dialflo, and frozen. If the Sephadex G-75 column (Column IV) is developed with distilled water according to the procedure of Borders and Raftery (4), the loss in activity is high as NaCl is essential to stabilize the purified enzyme. NaCl is also helpful to dissociate hyaluronidase from contaminant resulting in good separation. Hyaluronidase obtained from Column IV (Fig. 2) was free from β-N-acetylglucosaminidase and β-glucuronidase activities.

**Disc Gel Electrophoresis—**The purity of the enzymes was determined by disc gel electrophoresis changing both gel concentration and pH (the nine different gels showed no other major contaminating proteins). Testicular hyaluronidase obtained by the method of Soru and Ionescu-Stoian (3) and supplied by Worthington Biochemicals was compared with hyaluronidase obtained from Column IV which showed one major component (Fig. 3) on 7.5% gel at pH 4.3. One major peak was obtained from Column IV.

### Table I

| Procedure | Total protein | Specific activity | Total activity | Relative activity | Yield |
|-----------|--------------|------------------|----------------|------------------|------|
| Crude bull seminal plasma | 1,080 mg | 13 units/mg protein | 14,000 | 1.0 | 100 |
| (NH₄)₂SO₄ fractionation Separation of insoluble protein | 650 mg | 16 units/mg protein | 10,400 | 1.2 | 74 |
| Column I. Sephadex G-100 | 101 mg | 50 | 5,100 | 4 | 36 |
| Column II. DEAE-cellulose | 28 mg | 140 | 3,900 | 11 | 28 |
| Column III. Sephadex G-75 | 3.3 mg | 850 | 2,800 | 65 | 20 |
| Column IV. Sephadex G-75 | 1.7 mg | 1,300 | 2,200 | 100 | 16 |
| Column IV. Sephadex G-75 | 0.14 mg | 2,370 | 330 | 180 | 2 |

* One unit = 29 N.F. units.
Fig. 2. Elution profile of active fractions from Sephadex G-75 (Column IV). The column dimensions were 2.5 x 36 cm and 2.5-ml fractions were collected. The fractions marked by arrows had constant specific activity. Enzyme assays were performed using 0.05 ml of each fraction. O--O, protein; □-□, hyaluronidase.

band was also observed on sodium dodecyl sulfate gel electrophoresis (Fig. 4).

Properties

pH Optimum—Bull sperm hyaluronidase has an optimum at pH 3.8 with a broad plateau of activity between pH 3.5 and pH 8.0. The enzyme is inactive at pH 2.6 and has less than 10% activity above pH 8.0. Ram sperm hyaluronidase showed a somewhat different pH activity curve (13).

Cation Effects—Hyaluronidase from bull sperm has a requirement for cations. The effects of several mono- and divalent chlorides on enzyme activity are shown in Fig. 5. K+ and Na+ have a greater effect and at a wider range of concentration than Ca2+, Mg2+, and Mn2+, whereas, Co2+, Cu2+, and Zn2+ do not affect enzyme activity. Co2+ catalyzes substrate hydrolysis non-enzymatically. Zaneveld et al. (11) reported that Mg2+ and Ca2+ had no apparent effect on the activity of bull sperm acrosomal hyaluronidase at a fixed concentration and Fe2+ and Fe3+ decreased the enzyme activity. Using different concentrations of salts, our results showed that higher concentrations of ions inhibit enzyme activity.

Stability—Crude hyaluronidase at pH 7.0 is stable at room temperature for several days or frozen for several months. It is stable at 42° for 60 min, but loses 98% of the activity at 60° for 30 min. Pure hyaluronidase (Column IV) is not stable stored in solution at 4° for several weeks.

Kinetics—Kinetic studies performed at 37° with hyaluronic acid as substrate gave a $K_m$ of 3.7 mg per ml and $V_{max}$ of 2.4 μmol per min (Fig. 6).

Molecular Weight Determination—The mobility of the purified hyaluronidase (Column IV) compared to standards on sodium dodecyl sulfate gel electrophoresis (Fig. 4) gave a molecular weight of 62,000.

DISCUSSION

Hyaluronidase is involved in the dispersal of the cells of the cumulus oophorus of the ovum by dissolving the intercellular matrix which is composed of hyaluronic acid aiding the entry of the spermatozoa through this barrier. Recently, it has been shown that species-specific antibodies against hyaluronidase can be developed that may be useful in preventing gamete interaction (21, 22). In order to prepare species-specific antibodies pure hyaluronidase as obtained here would be very useful.

Testicular hyaluronidase is active at neutral pH by viscometric and turbidimetric methods of assay (23). Aronson and Davidson (24) and Gibian (25) showed that lysosomal hyaluronidase of rat liver has an optimum at pH 3.5 and is inactive above pH 4.5.
FIG. 4. Enzyme migration pattern on sodium-dodecyl sulfate gel electrophoresis using 30 μg of sample and 10 μg of each standard. A, enzyme obtained from Column IV; B, standards were: serum albumin, 68,000 mol wt; ovalbumin, 43,000 mol wt; pepsin, 35,000 mol wt; trypsin, 23,300 mol wt; lysozyme, 14,300 mol wt.

FIG. 5. Cation effects on enzyme activity were obtained using various chloride salts with changing concentrations. Substrate used in the assay was hyaluronic acid (Grade III, Sigma). □—□, Mg²⁺; △—△, Ca²⁺; ×—×, Mn²⁺; ○—○, K⁺; ●—●, Na⁺.

FIG. 6. Hofstee plot for the determination of $K_m$ and $V_{max}$. The action of bull sperm hyaluronidase on hyaluronic acid (Grade III, Sigma) was assayed using highly purified enzyme at 37°C in NaCl and 0.02 M sodium acetate buffer, pH 3.8.

Bull sperm hyaluronidase has a molecular weight of 62,000 and partially purified bull sperm acrosomal hyaluronidase has a molecular weight of 110,000 (11). This discrepancy may be due to a dimer of the partially purified acrosomal hyaluronidase.

Sperm hyaluronidase is 1.5 times more active than highly purified testicular hyaluronidase (4) and several hundred times more active than the partially purified bull sperm acrosomal hyaluronidase (11).

Sperm hyaluronidase from bull has an absolute requirement of NaCl for its activity. With hyaluronic acid of highest purity (Sigma, Grade I) or with less pure hyaluronic acid (Sigma, Grade III), sperm hyaluronidase has no activity in absence of NaCl. It is possible that Na⁺ has a direct effect on the enzyme as it has been suggested that cations prevent the dissociation of dilute solution of testicular hyaluronidase into inactive subunits (26). On the other hand, if the substrate contains chondroitin sulfate B, a potent inhibitor of hyaluronidase as a contaminant, then ionic strength and cations would reverse this inhibition (27, 28). We observed that the same batch of hyaluronidase showed markedly variable activity on different lots of hyaluronic acid prepared by Sigma. In contrast, lysosomal hyaluronidase does not require NaCl for optimal activity when hyaluronic acid...
prepared from the umbilical cords by the authors is used as the substrate (29, 30).

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Additions and Corrections

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In Yang, Chul-Hak, and Prakash N. Srivastava. Purification and Properties of Hyaluronidase from Bull Sperm

Page 79, first column, Summary, fourth line from the top, 2,370 Units; second column, 18th and 19th line from the bottom, 7.93 Units and 1 colorimetric Unit; and Page 80, second column, Table I, Units/mg protein and “one unit

All above units should read as milliunit instead of unit

Page 79, first column, Summary, second line from the bottom; Page 81, first column, ninth line from the bottom; and Fig. 6

V_{max} = 2.4 \text{ nmol/min}

In brief, following the definition of international unit as 1 \text{ pmol/min} (Page 79, column 2, first sentence in paragraph 4), the colorimetric units of hyaluronidase activity presented elsewhere in the paper are milliunits. V_{max} is 2.4 \text{ nmol/min}.

Vol. 250 (1975) 7593-7601

In Saier, Milton, H., Jr., Brigitte U. Feucht, and Michael T. McCaman. Regulation of Intracellular Adenosine Cyclic 3’;5’-Monophosphate Levels in Escherichia coli and Salmonella typhimurium. Evidence for Energy-dependent Excretion of the Cyclic Nucleotide

Page 7600, Table VII, column 3 “Intravesicular cyclic AMP”, line 4, 0 should read: 100

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