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STIMULATION OF BULL SPERM HYALURONIDASE BY POLYCATIONS

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Summary

The activity of bull sperm hyaluronidase (hyaluronate 3-glycanohydrolase, EC 3.2.1.36) is increased by the inclusion of polycations in the assay mixture. At pH 3.8, bovine serum albumin and histone give the greatest stimulation, while protamine sulfate, spermine, spermidine and hyamine 2389 stimulate to a lesser extent. Enzyme activity increases with serum albumin concentration to a nearly constant, high level at serum albumin concentrations greater than 1 mg/ml. Other stimulatory compounds show a similar concentration dependence except that inhibition of enzyme activity occurs at high concentrations of stimulator. The degree of stimulation depends on the pH, sample concentration and substrate concentration. Enzyme preparations with a low protein content give the greatest stimulation, while preparations with a high protein content show little stimulation. The concentration of serum albumin required for maximum stimulation increases with increased hyaluronic acid concentration. The results suggest that the stimulation of sperm hyaluronidase is nonspecific and results from an interaction of the polycation with hyaluronic acid. Since protein in the enzyme preparation substitutes for exogenous stimulator to a varying degree, serum albumin should be included in the assay mixture for sperm and testicular hyaluronidase to assure measurement of maximum enzyme activity.

Introduction

Sperm hyaluronidase (hyaluronate 3-glycanohydrolase, EC 3.2.1.36) disperses the cumulus cells which surround mammalian eggs and is thought to

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid.

participate in the removal of this cell layer during fertilization [1]. Because of its important function, this enzyme has been extensively studied. The properties of sperm hyaluronidase are similar to those of testicular hyaluronidase [2—4] and differ from those of lysosomal hyaluronidase [5,6]. In particular, the sperm and testicular enzymes have significant activity at neutral pH while the lysosomal enzyme is inactive above pH 5.0 [2,5,6].

Previous investigators have reported that the activity of hyaluronidase is increased when proteins or polyamines are included in the enzyme assay [7—9]. In a review, Meyer and Rapport [7] mentioned that serum, serum albumin and gelatin stimulate hyaluronidase activity. However, the source of enzyme and the assay conditions were not specified. Bernfeld et al. [8] reported the polyamines stimulate the activity of testicular hyaluronidase, particularly at low enzyme concentrations. Bovine serum albumin did not activate under their assay conditions. They suggested that the polycations increased hyaluronidase activity by preventing dissociation of the enzyme at low concentrations. More recently, Rogers and Morton [9] reported that hyaluronidase from bovine testes and guinea-pig sperm is stimulated by human serum albumin. They concluded that stimulation is specific for serum albumin and results from an interaction between the albumin and hyaluronic acid. Subsequent investigators have added serum albumin to the assay for sperm hyaluronidase to obtain increased sensitivity [10—12].

To help clarify the extent and nature of stimulation, the effect of serum albumin and other compounds on the activity of sperm hyaluronidase was reinvestigated. This report presents evidence that stimulation of sperm and testicular hyaluronidase is a nonspecific effect of polycations, including proteins. Stimulation appears to result from charge-charge interaction between the polycation and hyaluronic acid. The amount of stimulation depends on the protein content of the enzyme sample, the largest increase occurring with samples of low protein content. Therefore, serum albumin is necessary to maintain optimal assay conditions for sperm hyaluronidase.

Materials and Methods

Bovine serum albumin (recrystallized), bovine testicular hyaluronidase (type IV), hyaluronic acid (grade I), *N*-acetylglucosamine, spermine, spermidine, calf thymus histone (type II), protamine sulfate (salmon, grade II), protamine sulfate (herring, grade III) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were purchased from Sigma Chemical Co. Hyamine 2389 was purchased from J.T. Baker Chemical Co. All other chemicals were reagent grade.

Semen was collected using an artificial vagina and sperm fractions prepared as previously described [13]. Briefly, sperm were washed twice by centrifugation through 1.3 M sucrose containing 0.15 M NaCl. The washed sperm were resuspended in 0.15 M NaCl/5 mM Hepes, pH 7.0, and homogenized. The sperm homogenate was fractionated by layering on a discontinuous gradient consisting of 1.3 M sucrose/0.15 M NaCl and 1.7 M sucrose/0.15 M NaCl and centrifuging for 3 h at 27 000 rev./min in a SW27 rotor. The clear, soluble materials remaining at the top of the gradient was removed prior to collecting

membrane fractions. Plasma membrane, outer acrosomal membrane and pellet fractions were then removed from the gradient, concentrated by centrifugation and finally resuspended in 0.15 M NaCl/5 mM Hepes. Samples were stored frozen until assayed for hyaluronidase.

Hyaluronidase activity was assayed in a reaction mixture which contained, in 0.1 ml, 0.05 M acetate, pH 3.8, 0.15 M NaCl, 0.625 mg/ml hyaluronic acid and varying amounts of enzyme preparation and stimulator. After mixing the other assay components, the reaction was initiated by the addition of 0.05 ml of substrate solution in buffer and incubated for 2 min at 37° C. The reaction was terminated by the addition of the borate solution used to determine product, as described below. Each assay was performed in duplicate or triplicate with appropriate blanks. When the reaction pH was varied, the buffers used were glycine, pH 2.5—3.0; acetate, pH 3.5—5.5; cacodylate, pH 6.0—7.0 and Hepes, pH 7.0—8.0.

The free *N*-acetylglucosamine end groups produced by hyaluronidase action were estimated as described by Reissig et all [14]. First, 0.05 ml of a mixture containing 2 vols. of 0.8 M borate, pH 9.1, and 3 vols of 0.1 M NaOH were added to each tube, and the tubes heated in a boiling water both for 3—6 min. After cooling, 0.6 ml of 1% *p*-dimethylaminobenzaldehyde in acid were added to each tube (see Ref. 15 for details). The tubes were heated for 20 min at 37° C, cooled and the absorbance measured at 585 nm. Standards of *N*-acetylglucosamine were used to convert absorbance to nmol of product.

In determining product, it was critical that the reaction mixture, after the addition of borate solution, be at pH 8.9 for optimum color production [14]. To accomplish this, the reaction mixture was neutralized by the addition of an appropriate volume of 0.1 M NaOH. This volume was determined separately for each pH. For pH 3.8, 0.03 ml of 0.1 M NaOH was required. In the final assay procedure, the NaOH and borate solutions were mixed in the appropriate ratio and the mixture used to stop the enzymic reaction. This procedure was chosen to minimize precipitation of protein when the reaction mixture was heated. When significant precipitation occurred, and with particulate samples, the tubes were centrifuged prior to determining the absorbance.

Protein was assayed by the method of Lowry et al. [15] using bovine serum albumin as standard.

Results

Stimulation of sperm hyaluronidase was studied using soluble and pellet fractions prepared from bull sperm as described by Zahler and Doak [13]. All assays were conducted in the presence of 0.15 M NaCl, which is near the optimum salt concentration for this enzyme [3]. As illustrated in Fig. 1, bovine serum albumin increased the rate of hyaluronidase activity. With soluble preparations (Fig. 1A), little or no activity was measurable in the absence of added albumin and extended incubation time (20 min) was needed to demonstrate activity. The addition of increasing concentration of serum albumin dramatically increased enzyme activity until a plateau was reached at approximately 1 mg/ml serum albumin. Higher concentrations of serum albumin cause only small and variable changes in enzyme activity. In contrast to the above results,

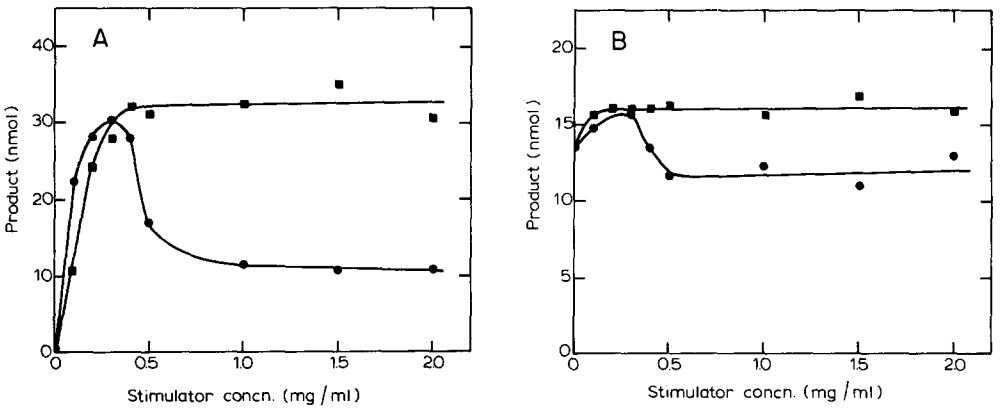

Fig. 1. Effect of serum albumin and histone on hyaluronidase activity in sperm fractions. Hyaluronidase activity was measured using 0.01 ml of enzyme preparations and the concentration of stimulator indicated. The stimulators used were bovine serum albumin (■) and calf thymus histone (●). (A) Soluble fractions, 0.94 mg protein/ml, 1.51 μmol/min per mg protein. (B) Pellet fraction, 10.5 mg protein/ml, 0.15 μmol/min per mg protein.

serum albumin produced only a slight activation of hyaluronidase activity in the pellet fraction (Fig. 1B).

Stimulation of sperm hyaluronidase in the soluble fraction did not require preincubation of the enzyme or the substrate with serum albumin. The same rate of enzymic activity was obtained when the reaction was initiated by addition of enzyme, substrate or serum albumin. Further, preincubation of the enzyme preparation at pH 3.8 for 10 min did not result in a significant decrease in activity. These results indicate that activation by serum albumin is rapid, and is not due to protection of the enzyme from denaturation.

In addition to serum albumin, hyarluronidase activity was stimulated by several other compounds (Fig. 1 and Table I). Increasing histone concentrations produced a sharp increase in hyaluronidase activity, followed by inhibition at higher concentrations. As with serum albumin, histone produced only slight

TABLE I

STIMULATION OF SPERM HYALURONIDASE

Hyaluronidase activity was assayed using standard conditions as described in Materials and Methods, with the indicated additions. The enzyme preparation was a soluble fraction from bull sperm and 0.01 ml was used for each assay. The specific activity of the enzyme preparation was 1.25 μmol/min per mg protein when assayed in the presence of 1 mg/ml bovine serum albumin. For the relative rate the rate of reaction for each stimulator relative to the rate of bovine serum albumin = 100.

Addition	Concentration (mg/ml)	Rate (nmol)	Relative rate
None	—	0.8	4
Bovine serum albumin	>0.5	21.6	100
Histone	0.3	20.8	96
Protamine sulfate (salmon)	0.5	15.5	72
Protamine sulfate (herring)	0.5	14.1	65
Hyamine 2389	0.5	5.4	25
Spermine	6	5.4	25
Spermidine	6	5.2	24

stimulation of hyaluronidase activity in the pellet fraction. Table I summarizes the effect of a variety of compounds on hyaluronidase activity using soluble enzyme. The greatest stimulation was obtained with serum albumin and histone, however a significant increase in activity was obtained using protamine sulfate, spermine, spermidine and hyamine 2389. Stimulation was also observed, qualitatively, when a slurry of DEAE-cellulose was added to the assay mixture. Of the compounds tested, only serum albumin and protamine sulfate (salmon) gave a plateau region of nearly constant, high activity. The other compounds showed a varying degree of inhibition at high concentrations.

The activity and degree of activation of sperm hyaluronidase was dependent on assay pH (Fig. 2). In the absence of added protein, hyaluronidase activity in the soluble fraction gave a pH optimum of about 6.0, with low enzyme activity. At optimal concentrations, both serum albumin and histone gave the greatest activation at low pH, resulting in optimal activity at pH 3.6. This pH optimum is in good agreement with the pH optimum reported for purified sperm [2] and testicular hyaluronidase [5,6]. Between pH 4. and 6, histone gave significantly higher activity than serum albumin, probably due to titration of serum albumin which has an isoelectric point in this pH range.

Hyaluronidase activity was studied as a function of enzyme concentration by varying the volume of enzyme preparation added to the assay (Fig. 3). In the absence of serum albumin, the increase in enzyme activity with sample volume was non-linear and little or no activity was detectable at low sample volumes. This effect was particularly pronounced with the soluble fraction (Fig. 3A). Serum albumin increased hyaluronidase activity at all enzyme concentrations with the most pronounced effect at low volumes and with the soluble fraction.

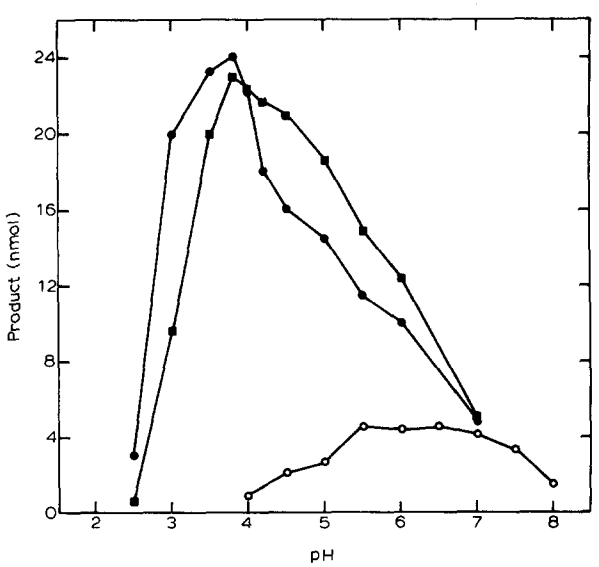

Fig. 2. The effect of pH on sperm hyaluronidase activity. Hyaluronidase activity was measured using 0.01 ml of a soluble fraction (0.96 mg protein/ml, 1.25 mol/min per mg protein). Stimulator concentrations were none (○), serum albumin, 1 mg/ml (●) and histone 0.5 mg/ml (■).

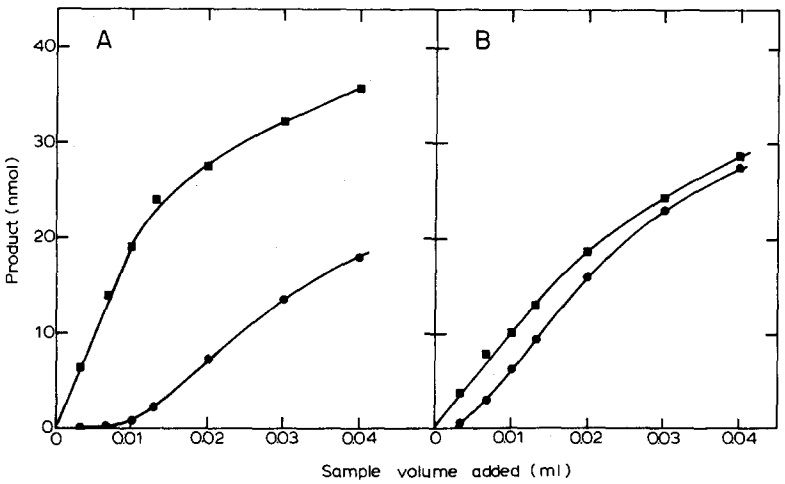

Fig. 3. Effect of enzyme concentration of hyaluronidase activity. Hyaluronidase activity was measured using the volume of enzyme preparation indicated. Stimulator concentrations were none (•) and serum albumin, 1 mg/ml (■). The enzyme preparations used were the same as for Fig. 1. (A) Soluble fraction; (B) pellet fraction.

As a result, the increase in rate with enzyme concentration was linear at low sample volumes.

When serum albumin, histone or other stimulators were added to hyaluronic acid solutions at pH 3.8, a milky suspension rapidly forms. In addition, serum albumin concentrations which produce suboptimal activation produce less turbidity, suggesting that stimulation of hyaluronidase results from an interaction of serum albumin with hyaluronic acid. To test this possibility further, the rate of hyaluronidase was measured at several serum albumin concentrations, and three concentrations of hyaluronic acid (Fig. 4). The concentration of serum albumin required for optimal stimulation of hyaluronidase activity was

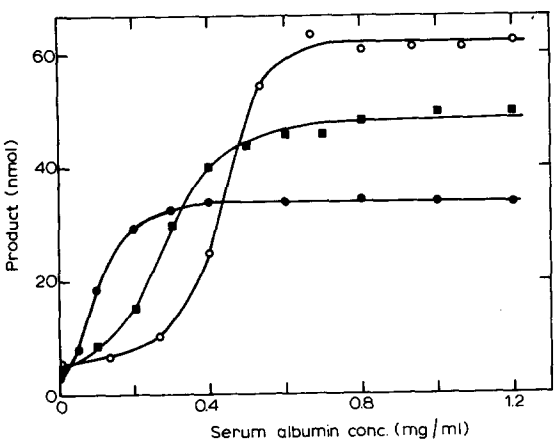

Fig. 4. Effect of substrate concentration on the stimulation of hyaluronidase activity. Hyaluronidase activity was measured using 0.01 ml of enzyme preparations and the concentrations of serum albumin indicated. Substrate concentrations were 0.3 mg/ml (•), 0.6 mg/ml (■) and 0.9 mg/ml (○).

lowest for 0.3 mg/ml hyaluronic acid and increased with increasing substrate concentration. These results are in agreement with those of Rogers and Morton [9] who reported that stimulation of testicular hyaluronidase depends on the ratio of serum albumin to hyaluronic acid.

Discussion

The results reported here confirm and extend previous observations on the stimulation of sperm and testicular hyaluronidase [7—9]. In agreement with Meyer and Rapport [7] and Bernfeld et al. [8], a variety of proteins and other compounds increase hyaluronidase activity when included in the assay. The greatest stimulation was obtained with serum albumin and histone, however, a significant increase in activity was found with protamines, polyamines, hyamine 2389, and DEAE-cellulose. The amount of stimulation depended on the concentration of stimulatory compound and the concentration of hyaluronic acid. All of the compounds which stimulate hyaluronidase activity are positively charged at the assay pH, suggesting that a primary requirement for stimulation is the interaction of a polycation with negatively charged hyaluronic acid. This conclusion is supported by comparison of the effect of serum albumin and histone as a function of pH. Both proteins give approximately the same amount of stimulation at pH 3.8, however, histone is more effective than serum albumin between pH 4 and 6. In the pH range, the net charge of serum albumin changes from positive to negative while that of histone remains positive.

It is well-known that serum albumin, in acid solution, complexes with hyaluronic acid to form a turbid suspension [16]. Our results suggest that such and interaction is responsible for the stimulation of sperm and testicular hyaluronidase. Stimulatory compounds produced a turbid suspension in the assay mixture, and the amount of tubidity correlated, qualitatively, with the amount of stimulation. In addition, when the concentration of hyaluronic acid in the assay was increased, the concentration of serum albumin required for optimal stimulation increased. These results support the conclusion of Rogers and Morton [9] that stimulation of testicular hyaluronidase results from an interaction between hyaluronic acid and human serum albumin. However, these authors suggest that stimulation is specific for serum albumin. This difference is likely due to differences in assay conditions.

Bernfeld et al. [8] have studied the stimulation of testicular hyaluronidase at pH 6.0. They found that the specific activity of hyaluronidase decreased when the enzyme was diluted. This loss of activity was prevented by the addition of several polycations, but not serum albumin, to the assay mixture. The failure of serum albumin to stimulate testicular hyaluronidase probably reflects the high assay pH. Based on their results, and previous work on β-glucuronidase [17,18], Bernfeld et al. [8] concluded that the decrease in hyaluronidase activity resulted from dissociation of the enzyme into inactive subunits, and that polycations stimulate by preventing this dissociation. Our evidence, however, indicates that stimulation is related to substrate concentration suggesting an interaction between hyaluronic acid and the stimulatory compound.

Since stimulation of sperm hyaluronidase is nonspecific, protein present in

the enzyme preparation can substitute for added serum albumin. This is evident from the difference in the amount of stimulation for soluble and pellet fractions and for different volumes of the same sample. In many cases, sample protein alone will not be sufficient to give optimal hyaluronidase activity, leading to an underestimation of the amount of enzyme. This was particularly true for the soluble fraction. therefore, to assure optimal assay conditions serum albumin should be included in the assay mixture for sperm and testicular hyaluronidase. This precaution will improve the measurement of hyaluronidase activity and aid in comparison of different sample preparations.

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