THE PREPARATION AND PROPERTIES OF A POLYSACCHARIDE SYNTHESIZED BY ACHROMOBACTER FISCHERI

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Received for publication July 20, 1951

The production of mucopolysaccharides by strains of groups A and C streptococci which are readily depolymerized by the hyaluronidases has prompted speculation as to whether this polysaccharide is limited to the capsular structure of streptococci. The possibility was considered that polysaccharide substrates for hyaluronidase may be formed by various bacteria as endocellular, exocellular, and capsular metabolic products.

It has been shown that polysaccharides isolated from *Bacillus megaterium* *Leuconostoc dextranicum*, *Rhizobium radicicolum*, and *Azotobacter chroococcum* (Madinaveitia and Stacey, 1944), failed to serve as substrates for hyaluronidase. More recently Warren (1950) found that a polysaccharide isolated from culture filtrates of a mucoid, capsulated strain of *Aerobacter aerogenes* was depolymerized by bovine testicular hyaluronidase.

During a study of the ability of the testicular hyaluronidases to depolymerize bacterial polysaccharides, it was found that when a suspension of the marine luminous bacterium *Achromobacter fischeri* was subjected to a temperature of 65 C to 70 C for 30 minutes, a marked increase in the viscosity of the suspension resulted. The addition of dilute, acidified horse serum (pH 4.2) to a small amount of the viscous material produced a voluminous, stringy clot. When the viscous material was incubated with either bovine testicular or streptococcal hyaluronidase, the formation of the clot was prevented. Further study revealed this factor to be a polysaccharide which served well as a substrate for hyaluronidase.

The work presented in this paper describes the partial purification of this hyaluronidase substrate as well as certain properties of the polysaccharide.

EXPERIMENTAL METHODS AND RESULTS

Culture. The culture of *Achromobacter fischeri*[1] originally isolated from a dead squid produced a smooth viscous, grayish to yellowish growth on 3 per cent NaCl nutrient agar containing 1 per cent glycerol. Luminescence was greenish which is typical for this organism. Attempts to demonstrate a capsular structure for *A. fischeri* have been unsuccessful (Johnson and Shunk, 1936; Warren, 1945).

Polysaccharide production. For the preparation of the polysaccharide, cultures of the organism were grown on the surface of agar in 32-ounce bottles. The bottles, each containing 200 ml of 3 per cent NaCl nutrient agar with 1 per cent glycerol and 0.5 per cent $CaCO_3$, were sterilized and the medium allowed to

[1] I am grateful to Dr. Frank H. Johnson of Princeton University for his kindness in sending me a strain of *Achromobacter fischeri*.

solidify with the bottle in a horizontal position. The bottles were heavily inoculated with a 18 to 20-hour culture of *A. fischeri* and incubated at 23 C to 25 C for 24 hours. With the aid of a sterile camel's hair brush the cells were carefully taken up in 3 per cent NaCl and aerated vigorously for 10 minutes. The cells were centrifuged and washed once with a large volume of the salt solution. They were then resuspended in 0.1 M sodium acetate buffer pH 6.0 and heated in a water bath to 65 C to 70 C for 30 minutes. A marked increase in the viscosity of the suspension resulted.

Partial purification of polysaccharide. The viscous suspension, clarified by filtration through "hyflo supercel" (Johns, Manville), was treated with two volumes of acetone. A white stringy precipitate formed immediately which remained in the ice box overnight. The precipitate was separated by centrifugation and the supernatant fluid discarded. The crude polysaccharide was dissolved in distilled water and reprecipitated by the addition of 8.5 per cent sodium acetate and 1.5 volumes of a 95 per cent ethyl alcohol. A total of three reprecipitations was performed in this manner. The final precipitate was dissolved in distilled water and dialyzed against frequent changes of distilled water for 48 to 72 hours at 4 C and then freeze-dried. Between 80 mg and 110 mg of a cream-white, amorphous substance were obtained from 20 bottles of culture medium.

Chemical analyses. The polysaccharide produced highly viscous solutions and gave strongly positive Molisch reactions. Negative protein tests were obtained with the biuret, Hopkins Cole, and trichloracetic acid reagents. Reducing substances were not present as shown by negative Fehling and Benedict tests. The nitrogen content of the polysaccharide as determined by the micro-Kjeldahl was 7.53 per cent; sulfur was not detectable.[2]

Depolymerization of the polysaccharide. Bovine testicular hyaluronidase prepared by the method of Hahn (1943), and crude group A *Streptococcus pyogenes* hyaluronidase were used in the depolymerization studies. The testicular and streptococcal enzymes possessed 650 and 20 turbidity reducing units per mg (TRU/mg), respectively, as determined by the assay procedure of Kass and Seastone (1944). A turbidity value of 40 scale divisions on the Klett-Summerson photoelectric colorimeter (red filter no. 66) was produced by the interaction of 0.4 mg of polysaccharide and acidified horse serum. The depolymerization of polysaccharide by hyaluronidase was determined by a turbidimetric assay method described previously (Warren *et al.*, 1948).

In the first experiment an example is given of the depolymerization of *A. fischeri* polysaccharide by active and heat inactivated (60 C—1 hour) testicular and streptococcal hyaluronidase.

It is evident from this experiment that heat-inactivated hyaluronidase, as shown by the inability of the enzyme to reduce the turbidity of vitreous humor potassium hyaluronate, was still capable of attacking to a certain extent the polysaccharide substrate (figure 1). However, heat-inactivated streptococcal enzyme did not depolymerize the substrate (figure 2). Since it has been shown

[2] I am indebted to Mr. Thomas R. McCrea for these data.

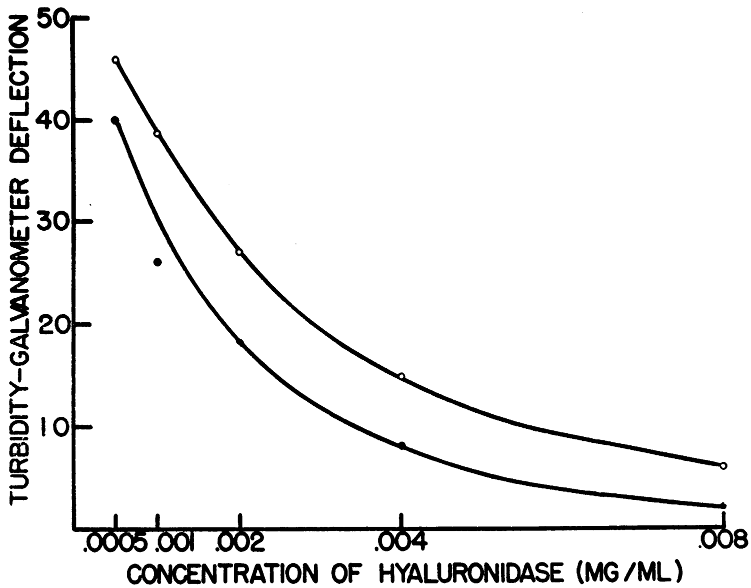

Figure 1. The depolymerization of *Achromobacter fischeri* polysaccharide by bovine testicular hyaluronidase. ●——● active enzyme. ○——○ heat inactivated enzyme. Each system consisted of 0.5 ml of 0.6 mg/ml polysaccharide and 0.5 ml of various dilutions of hyaluronidase. Incubated 30 min at 37 C. Reaction stopped and turbidity developed by addition of 3 ml of 0.5 M acetate buffer, pH 4.2, and 1 ml of 1:10 acidified horse serum pH 4.2. Read in a Klett-Summerson colorimeter; red filter no. 66.

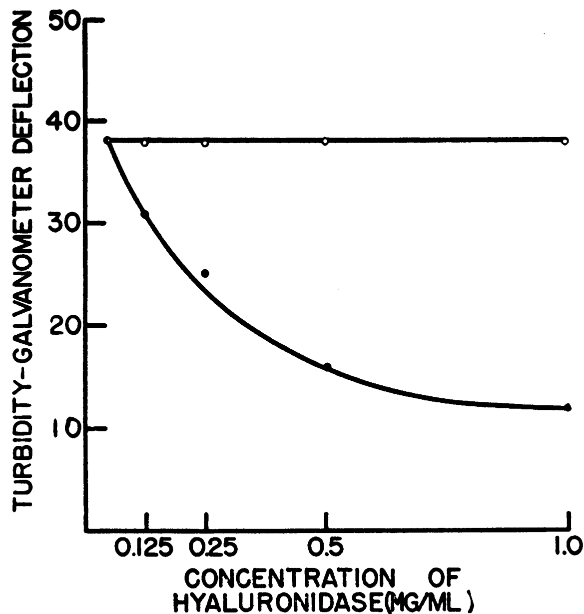

Figure 2. The depolymerization of *Achromobacter fischeri* polysaccharide by group A *Streptococcus pyogenes* hyaluronidase. ●——● active enzyme. ○——○ heat inactivated enzyme. (See figure 1.)

that several enzymes are involved in the breakdown of mucopolysaccharides by hyaluronidase preparations of the mammalian testes (Hahn, 1945), it is probable that the results obtained with the *A. fischeri* polysaccharide are due to enzymatic factors or nonenzymatic components of the testicular "hyaluronidase" complex not usually associated with the breakdown of potassium hyaluronate.

Comparative depolymerization studies with vitreous humor potassium hyaluronate and A. fischeri polysaccharide. Since it was apparent that the kinetics of enzymatic depolymerization of *A. fischeri* polysaccharide were somewhat different from those of potassium hyaluronate, it was considered of interest to compare the

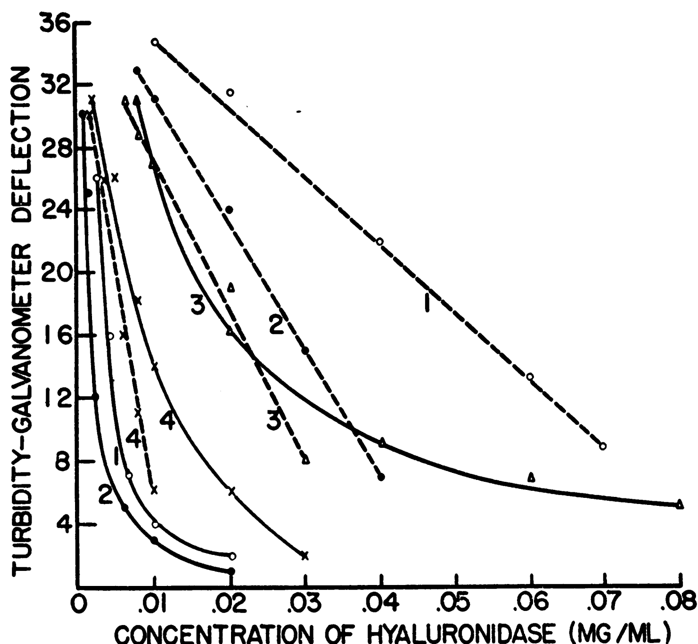

Figure 3. Comparison of the depolymerization of vitreous humor potassium hyaluronate and *Achromobacter fischeri* polysaccharide by varying concentrations of four testicular hyaluronidase preparations of different purity. The solid lines represent the *A. fischeri* polysaccharide; broken lines, potassium hyaluronate. The numbers denote the enzyme preparation: 1—205 TRU/mg; 2—650 TRU/mg; 3—940 TRU/mg; 4—2,250 TRU/mg.

rates of depolymerization of the two substrates with several hyaluronidase preparations of different purity. It was of special interest to determine whether the "hyaluronidase" complex involved in the breakdown of the polysaccharide increased in potency with the purification of hyaluronidase.

Four testicular hyaluronidase preparations ranging in potency from 205 TRU/mg to 2,250 TRU/mg were compared, the same enzyme dilutions and concentrations of substrate being employed in this study (figure 3). The enzyme preparation containing 2,250 TRU/mg was purified by the method of Tint and Bogash (1950). As might be expected, a linear correlation was found between the enzymatic activity of the various enyzme preparations as measured by the

depolymerization of the purified potassium hyaluronate. The reaction rates were dependent on the hyaluronidase concentration. This linearity was unlike the type of reaction curve obtained with *A. fischeri* polysaccharide. Although a more rapid rate of depolymerization was obtained with polysaccharide than with the hyaluronate, the reaction rates were relatively independent of the hyaluronidase concentration. No entirely satisfactory explanation is available for these results. However, the results are further evidence that the testicular hyaluronidases which attack hyaluronate are not identical with the "hyaluronidase" complex which depolymerizes the polysaccharide.

Effect of hyaluronidase on the luminescence and growth of A fischeri. It has been demonstrated by Meyer (1946) that lysozyme hydrolyzes a water insoluble polysaccharide which exists within the bodies of certain bacteria. In view of the extraction of a hyaluronidase-sensitive polysaccharide from *A. fischeri,* it seemed of considerable interest to extend these studies to include observations on growth and luminescence in the presence of testicular hyaluronidase. The possibility was considered that the polysaccharide of the intact cell might be available for enzymatic hydrolysis by hyaluronidase and an inhibition of luminescence or respiration might result.

The cells of *A. fischeri* were taken up in sterile 3 per cent NaCl solution, centrifuged, and washed twice with a large volume of salt solution. The bacteria were then resuspended in a solution consisting of equal parts of 3 per cent NaCl and M/4 phosphate buffer pH 7.3. The cells in 1 ml amounts were distributed in duplicate in a series of tubes (100 by 13 mm). A hyaluronidase preparation, possessing 2,250 TRU/mg and previously diluted to 1 mg per ml with NaCl-buffer, was sterilized by sintered glass filtration. To each tube was added 1 ml of serial dilutions of the hyaluronidase. The tubes were shaken and incubated for 2 hours at 25 C after which readings of luminescence were made in the dark room with dark-adapted eye.

The results demonstrated that testicular hyaluronidase did not inhibit the luminescence of *A. fischeri*. Furthermore, luminescence is actually increased in the presence of high concentrations of hyaluronidase. This is undoubtedly due to a nutrient substrate contributed by the testicular hyaluronidase preparation.

An experiment to determine the effect of hyaluronidase on the growth of *A. fischeri* was conducted in the following way: a series of test tubes containing 5 ml of NaCl-buffered infusion broth, pH 7.5, and hyaluronidase in varying concentrations was prepared. The concentrations ranged from 350 TRU/ml of broth to 3 TRU. All tubes, including a control tube without hyaluronidase, were seeded with 0.05 ml of a 20-hour culture of *A. fischeri* and incubated at 23 C to 25 C for 36 hours.

In the experiment previously described, no apparent inhibition of growth or luminescence of *A. fischeri* was observed. These results are in accord with previous observations concerning washed cell suspensions and hyaluronidase. It is, therefore, probable that the polysaccharide substrate is an endocellular metabolic product. Failure to inhibit growth or luminescence with hyaluronidase might

indicate that the enzyme does not have access to the polysaccharide in the intact bacterial cells.

SUMMARY

A polysaccharide extracted from the cells of the luminous marine organism, *Achromobacter fischeri*, has been shown to serve as a substrate for testicular and streptococcal hyaluronidase.

The isolation and partial purification of the polysaccharide are described.

The ability of heat inactivated hyaluronidase to show some depolymerization of the polysaccharide substrate emphasizes the differences between the "hyaluronidase" complex which attacks potassium hyaluronate and those which are concerned with the breakdown of *A. fischeri* polysaccharide.

While the work concerned itself mainly with the polysaccharide of the luminous organism, a comparison made with vitreous humor hyaluronate indicated that the activity of testicular hyaluronidase towards hyaluronate did not appear to be proportional to its activity towards *A. fischeri* polysaccharide.

The inability of testicular hyaluronidase to inhibit the growth or luminescence of *A. fischeri* may indicate the existence of the polysaccharide as an endocellular constituent of the cells.

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