THE DEPOLYMERIZATION OF BACTERIAL POLYSACCHARIDES BY HYALURONIDASE PREPARATIONS

GEORGE H. WARREN AND JANE GRAY

Wyeth Institute of Applied Biochemistry, Philadelphia, Pennsylvania

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It has been demonstrated that some strains of *Aerobacter aerogenes* and *Achromobacter fischeri* synthesized relatively large amounts of a mucopolysaccharide which was depolymerized by hyaluronidase (Warren, 1950, 1951). This synthesis is of special interest because the bacterial mucopolysaccharide (hyaluronic acid) depolymerized by hyaluronidase has been generally assumed to be limited to the capsular structure of mucoid strains of Groups A and C streptococci. It was thought of interest to study other bacteria as to their ability to produce polysaccharides which are depolymerized by hyaluronidase, and the relation of the synthesis of the polysaccharides to some environmental factors.

EXPERIMENTAL METHODS AND RESULTS

Cultures. In screening 60 cultures of various species of bacteria for hyaluronidase substrates the following organisms were found to produce polysaccharides which were depolymerized by the enzyme: *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Vibrio comma, Serratia marcescens,* and *Sarcina lutea.* All cultures studied were strains that had been maintained in a laboratory stock culture collection for several years on nutrient agar slants.

Isolation of crude polysaccharide material. The procedure used in following the polysaccharide concentration of cultures during growth was as follows: The organisms were grown on the surface of agar in Roux bottles. Each bottle contained 200 ml of 0.5 per cent NaCl nutrient agar with 1.0 per cent glycerol, and the reaction was adjusted to pH 7.4. Following sterilization the bottles were seeded heavily with a 24 hour culture of each species of organisms and incubated at 37 C for 1 to 5 days. The cells were harvested carefully with 25 ml of 0.1 M acetate buffer, pH 6.0, centrifuged, and resuspended in 25 ml of buffer. The bacterial suspensions then were placed in a boiling water bath for 30 minutes, and after removing bacterial debris by centrifugation or filtration, the supernatant fluid containing the crude polysaccharide was collected and stored at 4 C.

Since it was found that the isolated bacterial polysaccharides which were attacked by hyaluronidase gave a fairly stable colloidal suspension with dilute acidified horse serum (pH 3.1) similar to that produced by hyaluronic acid, a turbidimetric procedure (Meyer and Palmer, 1936) was employed in titrating the polysaccharide. The optimum incubation period for the synthesis of the polysaccharides was usually between 72 and 96 hours. For standardization purposes it was found convenient to dilute the polysaccharide with acetate buffer so that a turbidity value of 100 scale divisions on the colorimeter (red filter no. 66) was produced by the interaction of 1.0 ml of polysaccharide, 0.5 ml of acetate buffer, and 4.0 ml of a 1:40 dilution of acidified horse serum. In general a 1:2 to 1:4 dilution of polysaccharide was necessary to obtain this turbidity.

The depolymerization of crude polysaccharides by hyaluronidase. Bovine testicular hyaluronidase possessing 1,400 turbidity reducing units per mg (TRU/mg) as evaluated by the assay method of Kass and Seastone (1944) was used in the depolymerization studies. Each standardized polysaccharide was combined with 0.2 mg per ml of active and heat inactivated hyaluronidase (100 C—30 minutes) and tested for depolymerization by a turbidimetric method described previously (Warren *et al.*, 1948).

In the first experiment, standardized bacterial polysaccharides were depolymerized by active and heat inactivated hyaluronidase (table 1). A significant reduction in turbidity of the various polysaccharides was produced by the enzyme, but heat inactivated hyaluronidase was still capable of attacking the polysaccharide substrates to a certain extent. These results are in agreement with those obtained in a previous study with *Achromobacter fischeri* polysaccharide (War-

TABLE 1

The depolymerization of crude bacterial polysaccharides by hyaluronidase

ORGANISM	INCUBATION PERIOD	PER CENT TURBIDITY REDUCTION	
		Active hyaluronidase	Heat inactivated hyaluronidase
	hours		
Escherichia coli	96	51	40
Pseudomonas aeruginosa	72	49	33
Proteus vulgaris	96	43	20
Vibrio comma	96	55	44
Serratia marcescens	72	40	29
Sarcina lutea	96	25	20

ren, 1951) and may be attributed to enzymatic factors or nonenzymatic components of the testicular "hyaluronidase" complex not usually associated with the depolymerization of hyaluronic acid.

Polysaccharide production by bacteria in media containing various carbohydrates. The stock culture medium without added carbohydrate was prepared and dispensed by methods previously described. Twenty-five per cent solutions of galactose, sucrose, and glucose were sterilized by Seitz filtration and aseptically added to the sterilized liquefied medium so that a final concentration of one per cent was obtained. The bottles were seeded heavily with 24 hour cultures of the various bacteria and incubated at 37 C for 72 to 96 hours. Growth was measured turbidimetrically with the Klett colorimeter. The cells were harvested, extracted, and assayed for substrate activity as described.

The effect of the carbohydrates on the production of the polysaccharides can be seen in table 2. The values were obtained from several experiments. With the possible exception of *E. coli* and *V. comma*, no relationship appears to exist between the growth of the organisms and their ability to produce the polysaccharides. The addition of one per cent glycerol had a stimulating effect on the growth and substrate production of both of these organisms. It is interesting to note that media to which no carbohydrate was added did not support the synthesis of polysaccharide by *E. coli*, *P. vulgaris*, or *V. comma*. The remaining 3 organisms showed a small but definite polysaccharide production. The results would indicate that the polysaccharide factors can be increased in the presence of carbohydrates. Of particular interest is the fact that the majority of organisms produced consistently high yields of polysaccharide when glycerol was added to the medium.

TABLE 2

Effect of carbohydrates on the production of bacterial polysaccharides

ORGANISM	GROWTH (KLETT READING)	CARBOHYDRATE ADDED (1%)	PER CENT TURBIDITY REDUCTION	
			Active hyaluronidase	Heat inactivated hyaluronidase
Escherichia coli	60	none	0	0
	63	glucose	14	10
	130	glycerol	44	34
	76	galactose	14	9
	33	sucrose	0	0
Pseudomonas aeruginosa	30	none	17	10
	91	glucose	48	42
	72	glycerol	44	32
	68	galactose	34	23
	36	sucrose	23	14
Proteus vulgaris	70	none	0	0
	56	glucose	34	21
	59	glycerol	41	23
	56	galactose	27	17
	120	sucrose	36	30
Vibrio comma	65	none	0	0
	85	glucose	12	10
	136	glycerol	59	46
	83	galactose	24	13
	68	sucrose	12	10
Serratia marcescens	47	none	29	16
	48	glucose	25	23
	35	glycerol	37	28
	124	galactose	39	23
	122	sucrose	48	35
Sarcina lutea	105	none	22	17
	136	glucose	28	19
	113	glycerol	22	22
	112	galactose	19	13
	130	sucrose	22	17

A comparison of the per cent turbidity reduction of standardized polysaccharides by active and "inactive" hyaluronidase showed a predominance of lower reduction values for the "inactive" enzyme. Furthermore, the per cent

difference in turbidity reduction between active and "inactive" hyaluronidases varied with the organism and the carbohydrate present.

Partial purification of polysaccharides. The bacterial cells, grown in the presence of 1.0 per cent glycerol, were harvested and extracted for crude polysaccharide by the procedure previously described. The crude polysaccharide was treated with two volumes of acetone. A white stringy to flocculent precipitate formed immediately which remained when kept in the ice box overnight. The precipitate was separated by centrifugation and the supernatant fluid discarded. The polysaccharide was dissolved in distilled water and reprecipitated by the addition of 10 per cent potassium acetate and 1.5 volumes of 95 per cent ethyl alcohol. A total of 3 reprecipitations was performed in this manner. The final precipitate was washed repeatedly with ether and absolute alcohol, dissolved in distilled water, dialyzed at 4 C against frequent changes of distilled water for 72 to 96 hours, and then freeze-dried. Between 60 and 170 milligrams of a cream white, amorphous substance were obtained from one liter of culture medium.

Chemical analyses. All the polysaccharide preparations were readily soluble in distilled water and gave a strongly positive Molisch reaction. A negative protein test was obtained with the biuret reagent. Reducing substances were not present as shown by negative Fehling and Benedict tests. Quantitative estimation of nitrogen by the micro-Kjeldahl technique gave values ranging from 6.5 per cent to 8.4 per cent for the various polysaccharide preparations. Total phosphorous determinations using sulfuric acid for digestion and molybdate reagent and aminonaphtholsulfonic acid for development of color yielded values ranging from 1.4 per cent to 3.9 per cent depending upon the sample. The polysaccharides gave a negative test for sulfur.[1]

Effect of hyaluronidases of different purity on the depolymerization of partially purified polysaccharides. It was considered of interest to determine whether a quantitative relationship existed between the activity of the hyaluronidase preparation and the rate of depolymerization of the bacterial polysaccharides. Three testicular hyaluronidase preparations ranging in potency from 195 turbidity reducing units per mg to 1,400 turbidity reducing units per mg and in concentrations of 0.2 mg per ml were used in the depolymerization studies. The polysaccharides were standardized and used as substrates in the turbidimetric assay procedure in accordance with the methods previously described. The results are shown in table 3.

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

TABLE 3

Depolymerization of partially purified bacterial polysaccharides by hyaluronidase preparations

SOURCE OF POLYSACCHARIDE	HYALURONIDASE PREPARATION TRU/MG	PER CENT TURBIDITY REDUCTION	
		Active hyaluronidase	Heat inactivated hyaluronidase
		0.2 mg/ml	0.2 mg/ml
Escherichia coli	195	59	29
	760	68	46
	1,400	68	63
Pseudomonas aeruginosa	195	50	0
	760	70	6
	1,400	65	25
Proteus vulgaris	195	87	49
	760	97	69
	1,400	95	83
Vibrio comma	195	80	36
	76	80	41
	1,400	74	60
Serratia marcescens	195	44	21
	760	49	29
	1,400	44	42
Sarcina lutea	195	37	11
	760	36	23
	1,400	37	31

It can be seen from table 3 that the rate of depolymerization of the polysaccharides by the three active hyaluronidase preparations was relatively independent of enzyme concentration. However, the inactivation of the same enzymes by heat resulted in turbidity reduction values which were a function of enzymatic activity. Although no entirely satisfactory explanation is available for these results, the observations appear to indicate that testicular hyaluronidases

possess both heat stable and heat labile factors which attack the polysaccharides.

SUMMARY AND CONCLUSIONS

Polysaccharides which are depolymerized by testicular hyaluronidase have been extracted from strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Vibrio comma*, *Serratia marcescens*, and *Sarcina lutea*.

Although glucose, galactose, and in most cases sucrose were suitable carbohydrate sources for the polysaccharides, consistently good results were obtained with glycerol. The bacteria synthesized little or no polysaccharide in the absence of carbohydrate.

Since the isolated polysaccharides are attacked by a heat labile and heat stable component of hyaluronidase preparations, a significant difference is evident between the hyaluronidase factors which attack hyaluronic acid and those which are concerned with the breakdown of the polysaccharides.

REFERENCES

KASS, E. H., AND SEASTONE, C. V. 1944 The role of the mucoid polysaccharide (hyaluronic acid) in the virulence of Group A hemolytic streptococci. J. Exptl. Med., **79,** 319–330.

MEYER, K., AND PALMER, J. W. 1936 On glycoproteins. II. The polysaccharides of vitreous humor and of umbilical cord. J. Biol. Chem., **114,** 689–703.

WARREN, G. H. 1950 The isolation of a mucopolysaccharide from *Aerobacter aerogenes*. Science, **111,** 437–474.

WARREN, G. H. 1951 The preparation and properties of a polysaccharide synthesized by *Achromobacter fischeri*. J. Bact., **62,** 779–784.

WARREN, G. H., DURSO, J. G., AND LEVIN, N. R. 1948 A modified turbidimetric method for the assay of hyaluronidase. Endocrinology, **43,** 48–51.