The Isolation and Characterization of a Hyaluronidase Produced by a Capsulated Strain of Group C Streptococcus

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SUMMARY: A hyaluronidase preparation was isolated by the methods of protein precipitation from the culture fluid of a capsulated group C streptococcus cultivated in a serum-free medium. The preparation was active in the mucin clot prevention and turbidimetric tests for hyaluronidase and rapidly destroyed the capsules of group A and group C streptococci. The enzyme resembled other streptococcal hyaluronidases in its reaction to pH changes but differed from these both in its thermolability and antigenic specificity.

Hyaluronidase activity in cultures of capsulated or hyaluronic acid producing streptococci has previously been detected only by prolonged incubation of these cultures, or their filtrates or supernatants, with hyaluronic acid (Pike, 1948*b*; Quinn, Seastone & Weber, 1953; Faber & Rosendal, 1954). Little is known of the properties of the agent responsible for this activity beyond the fact that it is thermolabile and filterable (Pike, 1948*b*). However, the sera of patients with group A streptococcal infections often contain antibody to group A hyaluronidase, although the infecting organism produces hyaluronic acid and little or no hyaluronidase *in vitro* (Quinn, Seastone & Weber, 1953). This is further evidence that traces of hyaluronidase are formed in ageing cultures of capsulated group A streptococci and are responsible for the destruction of hyaluronic acid. The relatively high hyaluronidase activity of a capsulated group C strain reported by MacLennan (1956) suggested that this enzyme might be concentrated by protein precipitation and its properties more fully examined.

MATERIALS AND METHODS

Source of streptococci. National Collection of Type Cultures (NCTC). Strain LM (NCTC 6176) is a capsulated, hyaluronic acid and hyaluronidase producing group C strain. Strain C7 (NCTC 4540) produces hyaluronidase to high titre (Rogers, 1944) and is not capsulated at any stage of growth (MacLennan, 1956).

Cultivation of streptococci. Cultures were made as described by MacLennan (1956). They were grown from heavy inocula, saline washed, in broth at 37°, with continual neutralization by sodium hydroxide. After unsuccessful attempts to obtain preparations with high hyaluronidase activity by ammonium sulphate precipitation of supernatants from cultures in the serum enriched medium PSB (MacLennan, 1956) serum was replaced by distilled water. The serum-free medium PB is thus identical with PSB in the content

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and concentration of nutrients other than those present in serum. Despite a 25 % decrease in growth in PB cultures hyaluronidase activity by the turbidimetric test (MacLennan, 1956) was greater than that of PSB cultures at all stages of growth. The non-specific hyaluronidase inhibitor present in serum (Hobby, Dawson, Meyer & Chaffee, 1941; McClean, 1942) probably lowers the activity of PSB cultures; it is unlikely, in view of Pike's (1948*a*) work with non-capsulated streptococci, that the actual production of hyaluronidase is influenced by serum.

Preparation of strain C7 hyaluronidase. The formation of this enzyme is enhanced by cultivating the non-capsulated strain C7 in the presence of hyaluronic acid (Rogers, 1944). Cultures were grown, therefore, for 24 hr. in 0.1 % hyaluronate enriched PB (Rogers, 1946) for 24 hr. with continual neutralization by 10 % sodium hydroxide. The cells were discarded after centrifugation and thiomersalate added to the supernatant to a concentration of 1/10,000. This preparation is 'crude C7 hyaluronidase'. Ammonium sulphate and ferric hydroxide purified C7 hyaluronidase were prepared from culture supernatants by the methods described by Rogers (1946). Both contained 1/10,000 thiomersalate.

Preparation of testicular hyaluronidase. Adult rabbit testes, after removal of fat, were finely minced with an equal volume of distilled water and the suspension centrifuged. The supernatant was diluted to an activity of 500 M.C.P. units/ml., dispensed in 1 ml. amounts into ampoules and dried from the frozen state.

Measurement of hyaluronidase and hyaluronidase antibody. In addition to the sensitive turbidimetric test for the detection of small amounts of hyaluronidase (MacLennan, 1956) the turbidimetric test of Humphrey & Jaques (1953) and the mucin clot prevention (M.C.P.) test of McClean (1943) were used.

Antibody to hyaluronidase was titrated by the method of McClean (1943) using the M.C.P. test and by a turbidimetric method described below for the measurement of non-specific hyaluronidase inhibitors. All sera were heated at 56° for $\frac{1}{2}$ hr. before testing to destroy the non-specific, thermolabile inhibitor of hyaluronidase present in mammalian sera. Enzyme-antibody mixtures were allowed to react overnight at room temperature before measuring residual enzymic activity since neutralization of hyaluronidase by its antibody continues for some hours after mixing as noted first by Faber (1953). The activity of a serum was expressed as the number of M.C.P. units neutralized by a 1/20 dilution of the serum tested, and as the number of turbidity reducing units (T.R.U.) neutralized by 1 ml. of undiluted serum.

Measurement of non-specific hyaluronidase inhibitors. The inhibition of hyaluronidase by sodium azide, sodium fluoride, phosphorylated hesperidin and rehibin was measured turbidimetrically (Humphrey & Jaques, 1953) by Faber's method (1953) for the titration of hyaluronidase antibody. Inhibitor potency was recorded as the concentration in mg./ml. inhibiting 1 T.R.U. hyaluronidase.

Two series of enzyme dilutions in gelatin buffer were prepared to contain 0.5, 1, 1.5, 2, 3, and 4 T.R.U./ml. 0.2 ml. of aqueous dilution of inhibitor was

added to 0.8 ml. vol. of one series of dilutions and 0.2 ml. of water to the other. After $\frac{1}{2}$ hr. at room temperature 0.8 ml. of 0.08 % hyaluronic acid (Allen and Hanburys Ltd.) was added and the mixtures incubated for 30 min. at 37°. A 30 min. incubation was preferred to the 10 min. recommended by Humphrey & Jaques (1953) because of the weak activity of the strain LM enzyme. After incubation the tubes were chilled and 0.2 ml. of inhibitor solution added to the controls and 0.2 ml. of water to the experimental series immediately before precipitating residual hyaluronic acid with acid serum. This second addition of inhibitor was necessary because of the high colour of some inhibitor solutions which would otherwise cause differences in the colorimeter readings of control and experimental tubes.

Hyaluronidase antibody was also titrated by this method as a check on the M.C.P. test.

RESULTS

Thirty litres of a strain LM culture in PB were treated with thiomersalate, 1/10,000, after cultivation with continual neutralization for 7½ hr., the period found by trial for maximum hyaluronidase activity. The supernatant was siphoned off and 15 kg. ammonium sulphate added. The scum that formed was skimmed off, centrifuged and dialysed against running tap water until free from ammonium sulphate. A 150-fold decrease in volume was obtained. This preparation had an M.C.P. titre (20 min. incubation) of 40 units and a turbidimetric titre (30 min. incubation) of 19 T.R.U. This is the first demonstration of hyaluronidase activity in material from a culture of a capsulated streptococcus by conventional, quantitative methods of hyaluronidase assay. In the turbidimetric test the shape of the curve relating enzyme concentration to substrate destruction was very similar to that given by strain C7 hyaluronidase. The preparation, in common with strain C7 and testicular hyaluronidases, very rapidly decapsulated young cultures of strain LM and a group A strain. Enzymic activity was completely destroyed by 24 hr. at 37° and fell 50 % after 14 days at 22°. The preparation was therefore dried from the frozen state and stored at 2°. After unsuccessful attempts to purify the enzyme by adsorption on ferric hydroxide (Rogers, 1946) and calcium phosphate at pH 7.0 it was decided to characterize the enzyme in the impure concentrate in the hope that information thus acquired might suggest means of increasing the amount of hyaluronidase produced in culture. Purification could then be attempted on more active crude material.

The properties examined were the pH optimum and inactivation, the influence of temperature on stability, antigenicity and inhibition by fluoride, azide, phosphorylated hesperidin and rehibin. These properties were compared with those of the hyaluronidase produced in large quantities by the non-capsulated group C streptococcus, strain C7.

pH optimum and pH inactivation

It was not possible to use standard methods of hyaluronidase assay for enzyme-substrate mixtures over a wide range of pH values. McIlvaine buffers, M/30 with respect to the phosphate component, were found to maintain the

original pH of enzyme + substrate mixtures during enzymic destruction of the substrate at 37°. The sodium chloride content of the buffers was M/3 which gave optimal turbidity on precipitation of hyaluronic acid with acid serum at 0°.

The reaction mixture comprised 0.2 ml. aqueous enzyme dilution, 1.8 ml. M/30 McIlvaine buffer, M/3 with respect to NaCl and 2.0 ml. aqueous hyaluronic acid solution, 0.4 mg./ml. The enzyme was added last. Reaction mixtures with buffers of various pH values were prepared in triplicate, one series being used to confirm that the measured pH of the mixtures did not change during incubation at 37° for 30 min. The residual hyaluronic acid after incubation was precipitated at 0° with 4 ml. acid serum in the remaining two series of reaction mixtures. Turbidities were developed for ½ hr. at room temperature before reading on the Evans' Electroselenium Co. Ltd. (EEL) colorimeter, filter 622. Calibration curves were prepared from dilutions of the standard hyaluronic acid solution at each pH and from these curves the amount of hyaluronic acid destroyed was read off. At pH 7.0 turbidity was proportional to polysaccharide concentration and the amount of hyaluronic acid destroyed was proportional to enzyme concentration. The enzyme concentrations used were such that a slight change in concentration produced a large change in substrate destruction.

The pH optimum of strain LM hyaluronidase was compared with that of a strain C7 enzyme, dilutions having similar activity at pH 7.0 being used. Both were optimally active at pH 6 with a rapid fall below 5.6 and above 6.6 (Fig. 1). This observation agrees with those of Hale (1944) and Rogers (1948) for streptococcal hyaluronidases.

To measure pH inactivation enzyme preparations were adjusted to a series of low and high pH values by the addition of strong acid and alkali respectively. After ½ hr. at room temperature samples were neutralized with dilute acid or alkali and diluted to a constant volume. Residual enzymic activity was measured on 0.2 ml. samples by the method described for pH optimum measurements, using buffer at pH 7.0. Both enzymes lost activity greatly below pH 5 and above pH 8.

The influence of temperature on streptococcal hyaluronidases

There is no doubt that the streptococcal and pneumococcal hyaluronidases are more thermolabile than the testicular enzyme (Harris & Harris, 1950; Meyer & Rapport, 1952).

Thermolability was determined by heating 0.5 ml. vol. of aqueous enzyme dilutions at various temperatures for 15 min. and measuring residual activity on 0.2 ml. samples incubated with buffered substrate at pH 7.0, for 30 min. at 37°. As an additional precaution 0.4 % gelatin was included in the buffer since Rogers (1948) reported that gelatin, peptone and gum arabic protect hyaluronidases at ordinary temperatures. It was hoped by this means that enzyme preparations differing in protein content would be equally protected in the test.

The strain C7 enzyme, whether 'crude C7 hyaluronidase' or a protein concentrate prepared as described for strain LM, was destroyed between the

Fig. 1. The pH optima of strain C7 (○—○) and strain LM (●—●) hyaluronidases.

Fig. 2. The thermal destruction of strain C7 (○—○) and strain LM (●—●) hyaluronidases.

temperature limits 36.5° and 45° (Fig. 2). This result agrees with the observations of Harris & Harris (1950) on the rate of inactivation of pneumococcal and group A streptococcal hyaluronidases at 42°. Strain LM enzyme, on the other hand, was destroyed between the temperature limits 30° and 40°. This was confirmed several times; there is little doubt that this enzyme is more thermolabile than any previously described hyaluronidase.

The fall in hyaluronidase activity of strain LM cultures after growth has ceased (MacLennan, 1956) and in culture supernatants incubated at 37° in the presence of thiomersalate appeared now to be explained by the thermolability of the enzyme. The possibility remained, however, that the strain LM cultures, unlike those of strain C7, contained an agent that destroys hyaluronidase, for example a protease of wide substrate specificity such as is found in the culture supernatants of some group A streptococci (Elliott, 1945; Mycek, Elliott & Fruton, 1952). If this hypothetical agent resembled the protease described by Elliott in being more active at 37° than at 22°, then the rapid destruction of strain LM enzyme at 37° might be explained. To test this possibility the thermolability of strain LM hyaluronidase alone and in combination with strain C7 or testicular hyaluronidase was determined.

Table 1. *The thermolability of strain LM enzyme, alone, and in the presence of other hyaluronidases*

	Mg. hyaluronic acid destroyed/100 ml.			
	Unheated		Heated 40° for 15 min.	
	1	2	1	2
Hyaluronidase preparation				
1 vol. water + 1 vol. strain LM hyaluronidase	2.5	2.5	0	0
1 vol. water + 1 vol. strain C7 hyaluronidase (crude)	3	3	3	2.5
1 vol. water + 1 vol. testicular hyaluronidase	3.5	4	3.5	4
1 vol. C7 + 1 vol. LM	7	7	3	2.5
1 vol. testicular + 1 vol. LM	8	8	3.5	3

Strain LM enzyme and the two mixtures were heated at 40° for 15 min. The activity before and after heating was compared with that of the separate enzyme at concentrations equal to their individual concentrations in the mixtures. Table 1 shows that the enzyme activity of the heated mixtures could be attributed to that of the testicular or strain C7 enzyme components alone. A protease would presumably have destroyed all three enzymes indiscriminately. Since this did not occur the selective destruction of strain LM hyaluronidase is most probably due to its high thermolability.

The antigenicity of streptococcal hyaluronidases

Hobby *et al.* (1941) found that antiserum to pneumococcal hyaluronidase inhibited this enzyme but was inactive against streptococcal hyaluronidases. McClean (1943) reported that antiserum to strain C7 streptococcal hyaluronidase

neutralized other group C enzymes of types, 7, 20 and 21 but was inactive against group A, type 4. He suggested that the streptococcal enzymes were group specific but not type specific. Wenner, Gibson & Jaques (1951) have found that the hyaluronidases of streptococcal groups A, B and C are serologically distinct, whereas those of groups C and G are not.

Sterile filtrates (sintered glass filters) of strain LM and strain C7 enzymes were used to prepare antisera. The C7 enzyme preparations were purified by ammonium sulphate precipitation or by adsorption on $\text{Fe}(\text{OH})_3$. A crude preparation was also used. 0.5 ml. vol. were injected subcutaneously at weekly intervals into pairs of rabbits for 4 weeks and 1.0 ml. vol. by the same route after 3 weeks rest for a further 3 weeks.

Table 2. *The immunization of rabbits with streptococcal hyaluronidase preparations*

Antigen		Rabbit	No. of m.c.p. units neutralized by 1/20 antiserum							
Preparation	Unitage m.c.p./ml.		Bleeding							
		no.	Control	3rd	4th	5th	6th	7th	8th	9th
C7 enzyme (crude)	750	1	0	0	—	16	4	32	32	32
		2	0	1	—	32	16	64	64	64
C7 enzyme (ammonium sulphate)	1800	3	0	2	8	8	16	128	128	—
		4	0	1	32	64	16	64	64	—
C7 enzyme (ferric hydroxide)	500	5	0	0	—	2	2	8	32	16
		6	0	0	—	1	1	4	4	16
LM enzyme	22	7	0	0	—	0	0	6	6	3
		8	0	0	—	3	6	6	6	4

Table 2 reveals that all 3 strain C7 preparations produced a good antibody response as measured by the m.c.p. method. The $\text{Fe}(\text{OH})_3$ purified enzyme was the least effective, probably due to its low m.c.p. activity. Antisera to strain LM enzyme neutralized this enzyme but strain C7 antiserum did not neutralize strain LM enzyme or vice versa. This is the first report that hyaluronidases with different antigenic specificities are produced by streptococcal strains of the same group. Testicular and group A, type 4 streptococcal hyaluronidase were not neutralized by strain LM antisera either (Table 3). Since antisera to

Table 3. *The specificity of streptococcal hyaluronidase antisera*

Enzyme preparation tested	No. of m.c.p. units neutralized by 1/20 serum			
	Antiserum to C7 enzyme (ammonium sulphate)		Antiserum to LM enzyme	
	Rabbit 3	Rabbit 4	Rabbit 7	Rabbit 8
C7 Enzyme (ammonium sulphate)	80	40	0	1
LM enzyme	0	1	3	4
Group A, type 4 enzyme (crude)	0	1	0	0
Testicular enzyme	0	1	1	0

hyaluronidase produced *in vivo* by capsulated group A strains neutralize group A, type 4 enzyme it is clear that there is not a characteristic single hyaluronidase produced by capsulated streptococci irrespective of serological group.

Confirmation of the cross-neutralization tests was obtained from the turbidimetric method (Table 4). There was a slight neutralization of strain LM enzyme by the strain C7 antiserum.

Table 4. *The specificity of streptococcal hyaluronidase antisera in a turbidimetric test*

Enzyme preparation tested	No. of T.R.U. neutralized by 1 ml. undiluted serum			
	Antiserum to C7 enzyme (ammonium sulphate)		Antiserum to LM enzyme	
	Rabbit 3	Rabbit 4	Rabbit 7	Rabbit 8
C7 enzyme (ammonium sulphate)	600	610	0	0
LM enzyme	20	40	50	240

Hyaluronidase inhibitors

Weiner & Seastone (1951) observed that the normal process of capsule loss by group C streptococci in suspension was inhibited by azide and fluoride ions, although these did not prevent capsule destruction by streptococcal hyaluronidase. This implies that capsule loss may be caused by an enzyme other than hyaluronidase, but Weiner & Seastone were unable to isolate such an enzyme.

Since strain LM hyaluronidase is present in young capsulated cultures of this organism (MacLennan, 1956) and a protein concentrate of the enzyme can rapidly decapsulate streptococci there is little doubt that it plays a part in normal capsule loss. The action of fluoride and azide on this enzyme, and on strains C7 and testicular hyaluronidases, was examined by the turbidimetric test (Humphrey & Jaques, 1953) to measure inhibition in the manner suggested by Faber (1953).

Concentrations of 0.02 M-fluoride and azide in enzyme + inhibitor mixtures had no influence on enzyme action. It is possible that the decapsulating action of strain LM enzyme is inhibited by fluoride and azide; this was not investigated.

The action on strain LM and other hyaluronidases of rehibin, a polymerized derivative of gentisic acid (Hahn, 1952), and of phosphorylated hesperidin (Beiler & Martin, 1948), two inhibitors of testicular hyaluronidase, was examined. Both substances were powerful inhibitors of the testicular enzyme in the turbidimetric test and rehibin at fairly high concentration inhibited the strain C7 enzyme also (Table 5). Neither substance had any action on strain LM hyaluronidase but this may be due to the high protein concentration of this preparation since inhibition of the testicular enzyme diluted twofold in heated, inactive strain LM enzyme was considerably reduced. The inhibition of hyaluronidase by flavonoids such as hesperidin is not specific, other enzymes being inhibited (Rodney *et al.* 1950) and therefore the presence of other proteins

in hyaluronidase preparations might well reduce inhibition by combining with the inhibitor. The results with strain LM hyaluronidase are therefore inconclusive.

Table 5. *The inhibition of hyaluronidases in the turbidimetric test*

Enzyme preparation tested	Concn. of inhibitor in mg./ml. required to inhibit 1 T.R.U. enzyme	
	Rehibin	Phosphorylated hesperidin
Testicular enzyme	0.033	0.042
	0.036	0.055
Strain C7 enzyme (ammonium sulphate)	0.4	No inhibition
	0.37	at 0.5
Strain LM enzyme	No inhibition at 0.5	No inhibition at 0.5

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