A Ribonuclease of Group A Streptococci

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SUMMARY

A ribonuclease found in preparations of streptolysin S, unlike the streptococcal nucleases so far described, does not degrade DNA. It is only produced in significant amounts when RNA is present in the medium and is therefore an inducible enzyme. Unlike the previously described streptococcal enzymes it does not require divalent cations for activity. The molecular weight of the enzyme is about 14,000, its isoelectric point is 9.5 and it is highly heat-stable.

INTRODUCTION

The production of extracellular nucleases by a wide range of micro-organisms has been reported and these enzymes may be deoxyribonucleases, ribonucleases or less specific nucleases which are capable of degrading DNA and RNA. Deoxyribonuclease activity in the media from cultures of group A streptococci was first reported by McCarty (1948) and according to Wannamaker (1964) all 115 group A strains then examined produced extracellular deoxyribonuclease activity. Four enzymes are apparently involved, designated deoxyribonucleases A, B, C and D (Wannamaker, 1958, 1962). The B and D enzymes are capable of hydrolysing both DNA and RNA (Wannamaker, 1962; Yasmineh, Gray & Wannamaker, 1968) and should therefore be referred to as 'nucleases'.

The production of a true ribonuclease, without activity on DNA, by group A streptococci has not been described and it is likely that under the conditions of culture previously examined such an enzyme is not produced. This paper reports that a true ribonuclease can be induced in resting cocci by RNA. The enzyme was discovered during studies on the haemolysin streptolysin S which is prepared from resting cocci suspended in a medium containing RNA (Bernheimer, 1949). Its study was facilitated by the fact that it was first found in culture media of a variant organism deficient in extracellular deoxyribonucleases, but it has now been found in a wide range of serological types of group A streptococci. The characterization of the enzyme reported here has been performed on an unpurified product from this variant which contains none of the usual nuclease activity associated with streptococcal products. This variant appears to be the only reported deoxyribonuclease-deficient group A streptococcus.

METHODS

Strains of group A Streptococcus. Strain C 203 S, grown in Brain–Heart infusion broth, was originally obtained from Professor Alan W. Bernheimer. Strain BLACKMORE, grown in Todd–Hewitt broth, was obtained from Dr J. H. Humphrey, who originally obtained the organism from Dr Edgar W. Todd in 1946.

Commercial preparations. Todd-Hewitt broth and Brain-Heart infusion broth were from Oxoid Ltd. RNA grade V from Torula yeast, calcium bis-*p*-nitrophenyl phosphate, polyuridylic acid (5'), polyguanylic acid (5'), polyadenylic acid (5'), polycytidylic acid (5'), polyinosinic acid (5'), ovalbumin were from Sigma Ltd. Highly polymerized DNA from calf thymus, bovine pancreatic ribonuclease were from B.D.H. Ltd. 'Ampholine' carrier ampholytes were from LKB Ltd, Freund's complete adjuvant from Difco Ltd.

Ribonuclease assay. Ribonuclease activity was assayed according to the method of McDonald (1955) for partially purified preparations, but using 0.05 M-tris-HCl, pH 8.0, for the enzyme incubation. The enzyme activity unit is defined as the amount which produces an increase in O.D. of 0.1 at 260 nm. in the acid-soluble filtrate, after an incubation period of 10 min. at 25°.

Deoxyribonuclease assay. Deoxyribonuclease was assayed by the viscosimetric method of McCarty (1946), using 12 ml. viscometers with a flow time for water at 30° of 69 to 72 sec. One enzyme activity unit is that amount which causes a fall of 1.0 in relative viscosity in 20 min. under the standard conditions described.

Isoelectric focusing. An LKB 110 ml. column was used in conjunction with a 'Variopex' peristaltic pump and an 'Ultrorac' fraction collector (LKB). The methods used closely followed the manufacturer's instructions with Ampholine at 1 % and the enzyme sample added to the heavy component of the sucrose gradient. The column was cooled to 4° throughout the run. The initial voltage using the pH 3 to 10 gradient was 200 V, increased to 500 V after 2 h. for a further 18 h. For the runs using pH 8 to 10 Ampholine with an extended cathode region the final voltage was 350 V. The less dense solution in these runs contained 0.3 ml. Ampholine pH 8 to 10 in 50 ml. distilled water; the dense solution contained 1.2 ml. pH 8 to 10 Ampholine, 300 mg. arginine (HCl-free), 0.3 ml. ethylenediamine, 0.2 ml. triethanolamine in 50 ml. sucrose solution, 50 % (w/v). The bottom electrode solution was 20 ml. sucrose solution (50 % w/v) plus 1 ml. 40 % NaOH and 0.2 ml. ethylenediamine. The top electrode solution was 0.15 M-phosphoric acid. At the end of all runs the column was eluted at 1.5 ml./min. using the peristaltic pump, and 3 or 5 ml. fractions were collected. The pH of each fraction was measured before dialysis against cold distilled water for 24 h. in $\frac{1}{4}$ in. Visking tubing. The fraction volumes were then adjusted to 7.0 ml. with distilled water before ribonuclease assay.

Extracellular products of resting streptococci examined for ribonuclease activity

Four streptococcal products have been examined in this study:

(1) *Strain C 203 S SLS product.* This product was made according to the method of Bernheimer (1949) using his strain C 203 S.

(2) *Strain C 203 S non-haemolytic product.* As (1) but omitting the nucleic acid fraction from secondary medium in which the resting cocci are suspended.

(3) *Strain BLACKMORE SLS product.* This preparation was made according to Bernheimer's method but using strain BLACKMORE.

(4) *Strain BLACKMORE non-haemolytic product.* As (3) but omitting the nucleic acid fraction from the secondary medium.

RESULTS

Deoxyribonuclease and ribonuclease activities of the streptococcal products examined.

The four preparations were tested for the presence of both enzyme activities (Table 1). Strain BLACKMORE did not produce deoxyribonuclease but it produced a ribonuclease. The primary nutrient broth medium in which the organism had been cultured was also tested for deoxyribonuclease activity after 6 h. and after 16 h., but none was detected. These findings contrast with those of Bernheimer & Ruffier (1951), who found deoxyribonuclease activity in broth cultures of this strain and that it was also produced by the resting cocci.

Table 1. *Deoxyribonuclease and ribonuclease activity of streptococcal products*

Strain	No. of batches pooled	Deoxyribo-nuclease (units/mg.)	Ribo-nuclease (units/mg.)
C203S SLS product	8	70	10
C203S non-haemolytic product	4	60	10
BLACKMORE SLS product	8	—	13
BLACKMORE non-haemolytic product	5	—	—

Our strain was examined by Dr W. R. Maxted (Streptococcus Reference Laboratory, Central Public Health Laboratory, Colindale, London N. W. 9), who reported that it gave no positive serum opacity reaction (Krumwiede, 1954) nor a positive deoxyribonuclease test on a DNA plate. Both these tests are positive with the Reference Laboratory BLACKMORE strains and we conclude that our strain is a variant of the original BLACKMORE strain.

The ribonuclease activity in the C203S products is accompanied by deoxyribonuclease activity and may therefore be attributable to the presence of the enzymes B or D which break down both DNA and RNA.

The possibility that strain BLACKMORE produces a specific inhibitor of deoxyribonuclease activity was examined by re-assaying the C203S SLS product with the same concentration of BLACKMORE SLS product (2 mg./ml.). The deoxyribonuclease activity of the C203S SLS product was unchanged, hence no such inhibitor is present and the degradation of RNA by the BLACKMORE SLS product is due to a true ribonuclease.

Properties of the ribonuclease produced by strain BLACKMORE

Phosphodiesterase activity. The apparent BLACKMORE SLS ribonuclease activity is unlikely to be due to a phosphodiesterase since it does not degrade DNA. This was confirmed on incubating the enzyme (28 units/ml.) with bis-*p*-nitrophenyl phosphate (Koerner & Sinsheimer, 1957) when no phosphodiesterase activity was detected.

Molecular weight of the ribonuclease. The molecular weight of the enzyme was estimated using a calibrated Sephadex G-50 column (Andrews, 1964) 90 × 4 cm². The reference materials were glucagon, trypsin, ovalbumin and bovine pancreatic ribonuclease. The eluant was 0.1 M-NaCl containing 0.01 M-tris-HCl, pH 7.0. The elution volume of the enzyme was the same as that of pancreatic ribonuclease and its molecular weight was therefore approximately 14,000.

Isoelectric focusing. Strain BLACKMORE SLS, 15 to 20 mg., was dissolved in the heavy sucrose solution. Using a gradient of pH 3 to 10 the enzyme focused in the region of pH 9 to 10. In a gradient of pH 7 to 10 or pH 8 to 10 the pI of the enzyme could not be more accurately estimated as the pH 9 to 10 region of the gradient was never linear. However, by using a pH 8 to 10 gradient with an extended cathode region, as described in Methods, the pI was estimated to be 9.5 (Fig. 1). The recovery in the pH 3 to 10 experiment quoted was 50% and in the pH 8 to 10 run about 20%. The low recoveries may perhaps reflect an instability of the enzyme at its isoelectric point. Low recoveries of ribonuclease in some other cases also have not been explained.

Fig. 1. Isoelectric focusing of ribonuclease. Twenty mg. of BLACKMORE haemolytic product were applied in a pH gradient of 8 to 10 with an extended cathode region. Fractions of 5 ml. were collected. Conditions described under Methods. —, pH; ○—○, ribonuclease.

pH optimum for ribonuclease activity. The pH optimum for enzyme activity was determined by incubating 0.25 ml. of a solution of BLACKMORE SLS product (2 mg./ml.) plus 0.25 ml. 0.1 M Universal buffer (B.D.H.) with 0.5 ml. 0.5% RNA substrate. The enzyme is active in the pH range 7 to 10 with maximum activity at pH 8.0 (Fig. 2).

Heat stability. Strain BLACKMORE SLS product (2 mg./ml.) was heated in 0.05 M Universal buffer at pH 2.0, 4.0, 6.0, 7.0, 8.0 and 10.0 in a 100° water bath for 5 min. The samples were cooled and dialysed in $\frac{1}{4}$ in. Visking tubing overnight against distilled water at 4°, then assayed and compared with an unheated control sample. The results, in Fig. 3, show the enzyme to be stable at acid or neutral pH but more labile at alkaline pH.

Effect of divalent cations. Magnesium and calcium ions added (as chloride) from 5 to 100 mM to the standard assay system inhibited (Fig. 4). EDTA at 5 and 10 mM was without effect.

Activity of the ribonuclease on synthetic polynucleotides. Five synthetic substrates were tested with a ribonuclease preparation found to have a specific activity of 12.5

units/mg. in the standard assay system. The activity on poly C was 8.0 units/mg., on poly G 0.8 units/mg. and on poly U 1.3 units/mg. No activity was demonstrated on poly A or poly I.

Inducible nature of the enzyme. When resting streptococci were suspended in Bernheimer's secondary medium from which the nucleic acid fraction was omitted, ribonuclease production was not detected. The effect of increasing concentrations of nucleic acid fraction on the formation of the enzyme was investigated in the following

Fig. 2. pH optimum for ribonuclease activity of BLACKMORE haemolytic product (1 mg./ml.). Incubated in 0.05 M Universal buffer, with other conditions as for the standard ribonuclease assay.

manner. Bacteria from a 4 l. culture of strain BLACKMORE in Todd-Hewitt broth grown at 37° for 6 h. were washed twice with sodium phosphate buffered saline, pH 7.0 and suspended in 65 ml. of Bernheimer's secondary medium containing no nucleic acid fraction. This was divided into five equal portions and a different amount of nucleic acid fraction was added to each. The suspensions were then incubated at 37° for 2 h., centrifuged and the supernatants dialysed against cold distilled water and lyophilized. The residues were made up in 2 ml. normal saline and assayed for ribonuclease activity. Table 2 shows that ribonuclease was induced by nucleic acid fraction.

The presence of true ribonuclease activity in C203S products. The possibility that the ribonuclease described in strain BLACKMORE SLS product is also produced by strain C203S was examined indirectly by immunological methods. The nucleases of group A

streptococci so far described are immunologically distinct and it was considered likely that the presence of true ribonuclease in the C203S product would induce inhibiting antibodies in the rabbit specific for this enzyme. Two adult male New Zealand rabbits were immunized by intramuscular injection of 1 mg. C203S SLS product emulsified in

Fig. 3

Fig. 3. Loss of ribonuclease activity of BLACKMORE haemolytic product (2 mg./ml.) after heating for 5 min. at 100° in 0.05 M Universal buffer at different pH values.

Fig. 4

Fig. 4. Effect of Ca²⁺ (■) or Mg²⁺ (●) on ribonuclease activity of BLACKMORE haemolytic product (1 mg./ml.) assayed under the standard conditions.

Table 2. *Effect of nucleic acid fraction on formation of ribonuclease by resting cells*

Nucleic acid fraction (mg./ml.)	Ribonuclease produced (units/ml.)
5.0	14.0
1.0	11.0
0.1	5.0
0.01	0.8
—	0.2

Freund's complete adjuvant. After 15 days 1 mg. of the same product in sodium phosphate buffered saline, pH 7.0, was given subcutaneously. After 1 week the animals were bled by cardiac puncture. The effect of the immune sera thus obtained on the ribonuclease activity of BLACKMORE SLS product was tested as follows. Serum, 0.5 ml., was incubated at 37° for 1 h. with 0.5 ml. BLACKMORE SLS product (3.2 mg./ml.). Tris-HCl, 3.0 ml. of 0.1 M, pH 8.0, was added and the mixture centrifuged. The supernatant, 0.5 ml., was assayed for ribonuclease. Control sera were obtained from two

rabbits immunized with Freund's complete adjuvant alone. Rabbit 1 serum showed 100% inhibition and rabbit 2 71% inhibition compared with 0 and 4% inhibition by sera of control rabbits, which suggested that the C203S haemolytic product contains a ribonuclease identical with the BLACKMORE enzyme. This was confirmed by experiments using citrate and EDTA as inhibitors of the nucleases B and D. The ribonuclease activity in C203S non-haemolytic product was inhibited by citrate or EDTA, and this product therefore only contained nucleases B and D and no true ribonuclease activity. The ribonuclease activity of the C203S haemolytic product was only partly inhibited by citrate or EDTA, and we concluded that some true ribonuclease was present – as suspected from the inhibition of ribonuclease activity by antiserum to C203S haemolytic product. Thirty group A strains of different serological types have been investigated by this citrate-inhibition technique for production of true ribonuclease activity by resting cocci in the presence of RNA and all produced measurable amounts of enzyme. The sera of ten patients with streptococcal infections (demonstrated by high anti-streptolysin O titres) have been examined for neutralizing antibodies to the ribonuclease (A. G. Taylor, unpublished), but no inhibition was observed.

DISCUSSION

The release of extracellular ribonuclease when growth enters the stationary phase has been reported in a number of micro-organisms (Nishimura & Nomura, 1959; Yanagida, Uchida & Egami, 1964; Takai, Uchida & Egami, 1967), and Glitz & Dekker (1964) found that ribonuclease accumulates in the culture medium of *Ustilago sphaerogena* when RNA is the sole carbon source. This paper reports that a streptococcal ribonuclease is formed under the conditions used for streptolysin S production (Bernheimer, 1949) when maltose and RNA are present, but its formation with RNA as the sole carbon source or with other sources of nitrogen has not been investigated.

The absence of inhibiting antibodies to the enzyme in patients with high anti-streptolysin O titres suggests either that the enzyme is not formed *in vivo* or possibly it is not antigenic in the human. It is possible that its production by the organism under the culture conditions described is an adaptation to unfavourable environmental conditions when the digestion of polyribonucleotides in the medium can contribute to the nutrition of the organism.

Bernheimer (1949) has shown that the interaction of washed cocci and RNA results in the formation of streptolysin S, and it is possible that the enzyme described here may be important in the formation of the lysin.

The most striking difference between the ribonuclease described here and the streptococcal nucleases B and D is their different requirements for divalent cations. The dependence on such ions of both the ribonuclease and deoxyribonuclease activities of the enzymes B and D studied by Gray & Yasminéh (1968) was considered by them to be unusual and cited as further evidence for attributing both activities to a single enzyme in each case. The independence of such ions shown by the enzyme described in this paper is evidence that it is unrelated to those already described.

The specificity of the enzyme is similar to that of the ribonuclease activity of nuclease D (Gray & Yasminéh, 1968) in that it exerts a preferential action on cytidylic acid linkages but also degrades polyuridylic acid. Unlike nuclease D, however, it shows no activity on polyadenylic acid.

The molecular weight of 14,000 found here is within the range reported for a number of ribonucleases of bacterial and fungal origin which also show a high degree of base specificity (Barnard, 1969). They, like the one described here, also commonly show high stability to acid and heat and usually exhibit optimal activity at neutral or slightly alkaline pH.

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