BORN, G. V. R. (1952). *J. gen. Microbiol.* **6**, 344-351.

The Extracellular Bacteriolytic Enzymes of a Species of *Streptomyces*

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SUMMARY: A bacteriolytic protease produced by a species of *Streptomyces* is activated by manganese and other metals. This enzyme digests autoclaved *Bacterium coli* but not autoclaved *Staphylococcus aureus*, though it does digest 'proteins' extracted from staphylococci with hot alkali. A ribonuclease is produced by the *Streptomyces* sp. but it apparently takes no part in the lysis of *Staph. aureus*. During lysis autoclaved *Bact. coli* do not disintegrate but progressively diminish in size.

Culture filtrates of actinomycetes are known to lyse heat-killed bacteria, Gram-negative organisms being far more readily lysed than Gram-positive ones (Waksman, 1947; Welsch, 1947; Jones, Swallow & Webb, 1948). Tai & van Heyningen (1951) showed that a species of *Streptomyces* produced at least two proteolytic enzymes, the action of which could account for all the lysis of heat-killed Gram-negative organisms. One of these enzymes acted upon casein and the other had a greater affinity for protein(s) present in *Bact. coli*, but the caseinolytic enzyme may also have been bacteriolytic. The possibility that other, non-proteolytic, enzymes were concerned in bacteriolysis could not be excluded, although polysaccharases were apparently not involved. Heat-killed Gram-positive organisms were far less susceptible to lysis by the streptomyces enzymes. Jones *et al.* (1948) suggested that such organisms are lysed by proteolytic enzymes only after they have been made Gram-negative by the action of ribonuclease. In support of this suggestion they demonstrated that the filtrates of 35-day cultures of an *Actinomyces* sp. contained a ribonuclease and that lysis of Gram-positive organisms was brought about only with these filtrates and not with those obtained from 50-day cultures which did not contain ribonuclease. They did not show that ribonuclease actually participated in the lysis, nor did they show that the actinomyces ribonuclease changed the organisms from Gram-positive to Gram-negative during lysis. Dubos (1937) showed that pneumococci which had been lysed by crystalline trypsin to the extent of losing 75 % of their weight and 92 % of their nitrogen were still Gram-positive entities, and Welsch (1947) found that heat-killed staphylococci could be lysed by the enzyme system of a *Streptomyces* sp. and still remain Gram-positive. He also found that staphylococci made Gram-negative by treatment with bile salts at 60° were no more sensitive to his enzyme system than untreated controls.

It has now been found that, of the two streptomyces proteases demonstrated by Tai & van Heyningen (1951), the one which readily attacks the protein(s) present in *Bact. coli* is activated by manganese and other metals, while the

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caseinolytic protease is inhibited by manganese and more or less unaffected by other metals. The lysis of a heat-killed Gram-positive organism (*Staph. aureus*) is not activated by manganese, although the digestion of proteins extracted from this organism with hot alkali is activated by manganese. Attempts to purify and separate these two proteolytic enzymes of this *Streptomyces* sp. have met with only limited success. A ribonuclease has been demonstrated in the culture filtrate but it does not appear to participate in the lysis of staphylococci. During lysis of Gram-negative organisms there does not appear to be any dissolution of the organism, but rather a diminution in size.

METHODS

Lytic preparations and estimations of lytic activities

The culture of *Streptomyces* used was that of Tai & van Heyningen (1951), and their methods were used for producing lytic filtrates, concentrating the lytic enzymes and determining bacteriolytic and proteolytic activities. Bacteriolytic activity was estimated by measuring the percentage lysis, i.e. percentage decrease in turbidity, of a standard suspension of autoclaved *Bact. coli*, caused by the lytic preparation in 15 min. under defined conditions. Casein and autoclaved *Bact. coli* were used as substrates for the proteolytic enzymes, and the activity of these enzymes was estimated by measuring the increase in trichloroacetic acid-soluble products, formed during digestion for 20 min. under defined conditions, by the spectrophotometric method of Kunitz (1947).

Ribonuclease estimations were made by a method described by Born (1951). It consists in digesting yeast ribonucleic acid with samples of the unknown ribonuclease and of a standard preparation of the enzyme and comparing the turbidities that are produced when the undigested nucleic acid is precipitated with HCl under defined conditions. The method is not described since little use was made of it in the work to be reported here.

Other determinations. Bacterial counts were done in a Helber chamber with dark-ground microscopy. Nitrogen was determined by the micro-Kjeldahl method. Values of pH were determined electrometrically with a glass electrode. Manganese was determined by the method of Stockdale & Dexter (1938).

RESULTS

Comparison of the rates of lysis of Gram-positive and Gram-negative organisms

Suspensions of autoclaved *Bact. coli* and *Staph. aureus* (0.63 mg. dry wt./ml.) were treated with the streptomyces enzyme preparation or with crystalline trypsin (30 µg./ml.) for 30 min. at 37° and the rates of lysis compared. (According to Vendrely (1946) the protein contents of coliform bacilli and staphylococci are both about 75 % of the dry weight.) Table 1 shows that the streptomyces preparation lysed the Gram-negative organism 3 times as fast as the Gram-positive and that with trypsin the difference in rates was 2-fold.

When a lower concentration of the streptomyces enzymes acted upon these substrates for a longer time the difference in rates of lysis of Gram-negative

and Gram-positive organisms was not so great, as can be seen in Table 2. (These experiments were carried out with aseptic precautions.)

Table 1. *Lysis of autoclaved Bact. coli and Staph. aureus by a streptomyces preparation and by crystalline trypsin (30 min. at 37°)*

Substrate	Lysis (%) with	
	Streptomyces preparation	Trypsin
<i>Bact. coli</i>	77	50
<i>Staph. aureus</i>	24	25

Table 2. *Lysis of autoclaved Bact. coli, Staph. aureus and Strep. pyogenes by a smaller concentration of the streptomyces preparation than that used in the experiments shown in Table 1*

Time (hr.)	Lysis (%) of		
	<i>Bact. coli</i>	<i>Staph. aureus</i>	<i>Strep. pyogenes</i>
4	62	43	23
16	80	70	61
32	85	74	66

Table 3. *Effect of various metal ions (at 0.002M) on the proteolytic digestion of autoclaved Bact. coli by the streptomyces preparation*

Metal	Ratio:
	$\frac{\text{activity} + \text{metal}}{\text{activity} - \text{metal}}$
Manganese	3.44
Magnesium	2.10
Iron	1.45
Calcium	1.44
Copper	1.16
Cobalt	1.00
Zinc	0.98

Activation of bacteriolysis by metals

The effect of various metal ions on the proteolytic digestion of autoclaved *Bact. coli* by the streptomyces preparation was investigated. The effect of metals on the lysis of autoclaved *Bact. coli* could not be studied because addition of metal salts to the bacterial suspension caused variable changes in turbidity and interfered with the turbidimetric assay of bacteriolysis. Table 3 shows that manganese and some other metals had a considerable activating effect on the proteolysis of autoclaved *Bact. coli*.

Although emission spectroscopy (kindly carried out by Dr F. M. Brewer) showed no traces of manganese either in the streptomyces enzyme preparation or in the autoclaved *Bact. coli* substrate, various metal-binding compounds partially inhibited the action of the enzyme when no metals were added (Table 4). This inhibition may have been due to the removal of traces of other activating metals, such as magnesium or calcium, which were shown to be present.

Table 4. *Effect of metal-binding substances on the digestion of autoclaved Bact. coli by the streptomyces preparation*

Substance	Concentration (M)	Ratio: activity + substance activity - substance
Ethylenediamine tetra-acetate	0.005	0.40
Citrate	0.10	0.52
Thiolacetate	0.0025	0.54
Cysteine	0.0025	0.70
Oxalate	0.000125	0.80

Fig. 1

Fig. 2

Fig. 1. Activation of the proteolytic digestion of autoclaved *Bact. coli* by the streptomyces preparation in the presence of magnesium (A), manganese (B) and calcium (C).

Fig. 2. Effect of pH on the proteolytic digestion of autoclaved *Bact. coli* by the streptomyces preparation in the presence (A) and in the absence (B) of manganese.

Fig. 1 shows that the optimum concentration of manganese for the proteolysis of autoclaved *Bact. coli* was about 0.002M. Fig. 2 shows that manganese did not affect the optimum pH for this proteolysis. Dialysis for 24 hr. decreased the concentration of manganese from 0.002M to a level too low to estimate and completely reversed the activation. The effect of manganese on the digestion of several other proteins besides autoclaved *Bact. coli* was tested, with the results shown in Table 5. It is interesting to note that it was only the proteolysis of autoclaved *Bact. coli* which was activated, whereas the digestion of several other proteins, including casein, was inhibited. Other metals did not affect the digestion of casein.

Table 6 shows that manganese had no effect on the proteolysis of autoclaved *Bact. coli* by a crystalline protease of *Aspergillus oryzae* (kindly supplied by Dr W. G. Crewther; see Crewther & Lennox, 1950) or by crystalline trypsin.

At this stage it was clear that the manganese-activated enzyme in the streptomyces preparation was that which acted upon the protein(s) present in autoclaved *Bact. coli* and that several substrates from sources other than bacteria were not attacked by it. It was, therefore, interesting to find out whether this enzyme also acted on proteins of Gram-positive organisms.

Table 5. *Effect of manganese (0.002M) on the digestion of various proteins by the streptomyces preparation*

Substrate	Ratio:
	$\frac{\text{activity} + \text{Mn}}{\text{activity} - \text{Mn}}$
Autoclaved <i>Bact. coli</i>	3.44
Denatured fibrinogen	0.89
Denatured haemoglobin	0.70
Denatured serum albumin	0.60
Denatured globin (from haemoglobin)	0.53
Casein	0.40

Table 6. *Effect of manganese (0.002M) on the digestion of autoclaved Bact. coli by protease from Aspergillus oryzae and by trypsin*

Enzyme	Final concentration of enzyme ($\mu\text{g./ml.}$)	Ratio:
		$\frac{\text{activity} + \text{Mn}}{\text{activity} - \text{Mn}}$
<i>A. oryzae</i> protease	10	1.00
Trypsin	5	0.81

Suspensions of autoclaved *Bact. coli* and *Staph. aureus* (0.5 % dry wt./vol.) were treated with the streptomyces enzyme preparation with and without the addition of 0.002M manganese, for 20 min. in the case of *Bact. coli* and for 60 min. in the case of *Staph. aureus*. After these times the mixtures were precipitated with an equal volume of 10 % (w/v) trichloroacetic acid and the ultraviolet absorption spectra of the filtrates determined. These showed that whereas the digestion of *Bact. coli* was activated by manganese there was no activation in the case of *Staph. aureus* (Fig. 3).

These results suggested that Gram-positive organisms are lysed more slowly than Gram-negative organisms because the manganese-activated enzyme does not participate in the proteolysis of the former. The results do not necessarily mean that Gram-positive organisms do not contain the substrate of the manganese-activated enzyme. It is possible that the substrate is present but protected from the action of the enzyme. Some light is shed on this point by the following experiment. Autoclaved *Staph. aureus* suspensions were treated with 0.5N-NaOH for 20 min. at 56° and at 100° and centrifuged. The insoluble residues and the supernatants were neutralized and dialysed for 48 hr. against running tapwater and then digested with the streptomyces preparation with and without 0.002M manganese. The results are shown in Table 7. Proteolysis of fractions obtained at 56° was unaffected by manganese, but the fractions obtained at 100° were readily digested by the manganese-activated enzyme.

Whether this was due to the destruction or removal of an inhibitor or to an alteration of the protein was not decided.

Fig. 3

Fig. 3. Ultraviolet absorption spectra of trichloroacetic acid-soluble material liberated by the streptomyces preparation from autoclaved *Bact. coli* in the presence (A) and in the absence (B) of manganese, and from autoclaved *Staph. aureus* in the presence (C) and in the absence (D) of manganese.

Fig. 4

Fig. 4. Lysis of autoclaved *Staph. aureus* by ribonuclease (curve A from 0 to 1.5 hr.) and the streptomyces preparation (curve A from 1.5 to 2 hr.), and by the streptomyces preparation alone (curve B from 1.5 to 2 hr.).

Table 7. *Effect of manganese (0.002M) on the proteolysis of fractions obtained from autoclaved Staph. aureus by treatment with 0.5N-NaOH for 20 min. at 56° and 100°*

Temperature (°)	Fraction tested	Non- dialysable nitrogen in fraction (% of total)	Ratio: activity + Mn activity - Mn
56	Insol. residue	67.5	1.1
	Extract	32.5	1.1
100	Insol. residue	12.0	3.4
	Extract	88.0	3.9

Attempts to purify and separate the streptomyces proteases

Numerous attempts were made to separate and purify the streptomyces enzymes by salting out and adsorption, but they met with little success. An attempt was also made to take advantage of the fact that the manganese-activated enzyme was more strongly adsorbed on to autoclaved *Bact. coli* than the caseinolytic enzyme (see Tai & van Heyningen, 1951). The mixture of enzymes was adsorbed with autoclaved *Bact. coli*, the organisms were suspended in buffer and incubated at 37° until they had all dissolved, and the resultant solution was again adsorbed with autoclaved *Bact. coli*. When this process was repeated once more a 15-fold concentration of the manganese-activated enzyme relative to the caseinolytic enzyme was obtained. However, this procedure had no practical value as the yields of enzyme were extremely low, and although there was a separation of the enzymes there was no increase in their activity per unit weight of non-dialysable nitrogen.

Many attempts were made to purify the enzymes by fractional precipitation with organic solvents under controlled conditions of ionic strength, temperature, pH, protein concentration and solvent concentration. The two enzymes were not separated and at best a 4-fold increase in total enzyme activity was obtained when the enzymes were precipitated with 80 % methanol (v/v final concentration) at pH 4.0, temperature -5°, and at an ionic strength equal to that of tapwater (about 0.005). With 80 % methanol the yield of both enzymes was about 80 %; with 40 % methanol the same purification was obtained, but the yield of enzymes was 25 %. The great solubility of both enzymes and their associated impurities in concentrated $(\text{NH}_4)_2\text{SO}_4$ solutions and in ethanol, methanol, and acetone prevented their purification.

The role of ribonuclease in the lysis of Gram-positive organisms

The preparation obtained by precipitating the streptomyces enzymes with 80 % methanol at pH 4.0 had ribonuclease activity (per mg. non-dialysable nitrogen) equivalent to 1/2000th of that of a sample of crystalline pancreatic ribonuclease (kindly supplied by Prof. J. N. Davidson), and the yield of ribonuclease in this precipitate was at least 50 % of that contained in the crude streptomyces culture filtrates. Jones *et al.* (1948) suggested that the lysis of Gram-positive organisms by actinomyces proteases was preceded by the removal of the Gram-staining material by ribonuclease. This could not be confirmed with the streptomyces preparation. When suspensions of autoclaved *Staph. aureus* (containing 2-3 % of Gram-negative cells) were lysed to the extent of 50 % by the streptomyces preparation there was no change in the Gram-staining properties of the residual cells, and the trichloroacetic acid-soluble products absorbed ultraviolet light with a maximum at about 277 m μ . which is characteristic of proteins (see Fig. 3). On the other hand, when these cells were treated with small concentrations of crystalline pancreatic ribonuclease the trichloroacetic acid fraction absorbed intensely at 258 m μ . Crystalline ribonuclease did cause some decrease in the turbidity of a suspension

of autoclaved *Staph. aureus* (see Fig. 4), but the concentration of ribonuclease required to cause 25 % lysis was about 40,000 times as high as that in a streptomyces protease preparation that would cause the same amount of lysis in the same time.

Table 8. *Decrease in size of autoclaved Bact. coli cells during incubation with the streptomyces preparation*

(Temperature of incubation 37°.)

Time of incubation (min.)	Lysis (%)	Number of cells/ml. suspension	State of cells
0	0	2.5×10^9	Normal
20	38	2.6×10^9	—
45	58	2.6×10^9	—
60	63	2.5×10^9	Smaller, not elongated

Reduction in size of cells during bacteriolysis

When autoclaved *Bact. coli* cells were partially lysed by the streptomyces preparation there was apparently no dissolution of the cells but rather a diminution in size. Table 8 shows that after 63 % lysis there was no appreciable decrease in the total number of cells. Dubos (1937) obtained similar results when Gram-positive cells (heat-killed pneumococci) were treated with trypsin.

I am grateful to Dr W. E. van Heyningen for advice and help, and to the Medical Research Council for a Studentship in Research Methods.

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(Received 2 November 1951)