CCXVIII. PROTEINASE SECRETION AND GROWTH OF *CLOSTRIDIUM* *HISTOLYTICUM*

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YOUNG cultures of *B. fluorescens liquefaciens* quantitatively secrete a proteinase into the culture medium, as was found by Virtanen & Tarnanen [1931; 1932] and Virtanen & Suolahti [1937]. In previous work on the proteinase of *Cl. histolyticum* Weil & Kocholaty [1937] reached a similar conclusion.

Recently Maschmann [1938, 1] reported on a bacterial proteinase (*B. botulinus*, *B. Welchii*) which he considers to be in the "intracellularly acting proteinase of anaerobic bacteria", differing from the proteinase which is secreted by the bacterial cell. In a later publication on *Cl. histolyticum*, the same author [Maschmann, 1938, 2] concludes that this micro-organism produces three distinct proteinases. These are somewhat vaguely differentiated according to their actions on different protein substrates and their behaviour towards cysteine and other activators. Two of these enzymes are believed to be "extracellular" in character, being secreted in the early stages of bacterial growth and to be indifferent towards, or even inhibited by, cysteine and other common activators. The third enzyme is considered to be "intercellular", and to differ from the others in its ability to hydrolyse clupein but not gelatin, and to be activated by —SH compounds. Maschmann claims that the activation phenomena observed by us on culture filtrates of *Cl. histolyticum* are due to this intracellular proteinase, which is freed only by autolysis of the bacterial cell.

Our previous work on this micro-organism has been extended and the results are still at variance with the conclusions reached by Maschmann. In cultures of *Cl. histolyticum*, both the bacterial growth and the proteolytic activity (initial and full¹) of the cell-free bacterial filtrates, reach a maximum value within 24 hr. after inoculation. This is true regardless of the type of medium employed provided that the latter has pH near the neutral point. In all cases here investigated it was possible to activate the proteinase by means of Fe⁺⁺ in combination with sulphhydryl compounds. Thus, a proteinase activated by Fe⁺⁺-cysteine was found in filtrates from 6 hr. cultures, at a time when practically no bacterial autolysis had yet taken place. Similar activation was observed in filtrates prepared after incubation periods up to 12 days; Fe⁺⁺-cysteine likewise activated the intracellular proteinase obtained by destroying the bacteria themselves with a supersonic oscillator. On the basis of the results there appears no justification for assuming a difference between the intracellular proteinase and the proteinase secreted into the culture medium.

Cell-free filtrates obtained after incubation periods ranging from 12 to 48 hr. were found to hydrolyse both clupein and gelatin. In every case activation by

¹ By initial activity is meant the original proteinase activity without addition of activator; by full activity is meant the activity obtained in the presence of an Fe⁺⁺-SH activator. In the present work cysteine was used as the —SH source, under conditions described by Weil & Kocholaty [1937].

means of Fe^{++} -cysteine was observed. Maschmann states that the clupein-splitting enzyme is intracellular, and distinct from the gelatin-hydrolysing, extracellular enzymes. But its appearance in the culture filtrates (12 hr. incubation) before appreciable bacterial autolysis has occurred, indicates that it too is secreted and is probably identical with the gelatin-hydrolysing enzyme.

When cultures of *Cl. histolyticum* are incubated beyond the point at which maximum bacterial growth and proteolytic activity occur (24 hr.) a fairly rapid decrease in the activity of the proteinase takes place. This is not due to a temperature effect but appears to be related in some way to the autolytic processes. The stability of the bacterial proteinase depends also on the type of medium employed in the culture.

EXPERIMENTAL

The organism used was, as in previous work, a dissociated strain of *Cl. histolyticum* obtained from the American Type Culture Collection, No. 4872 [Hoogerheide, 1937]. The culturing was done as previously described, with the exception that the time of incubation was 24 hr. unless otherwise stated [Kocholaty & Hoogerheide, 1938]. The medium was in every case adjusted to pH 7.4 before inoculation. The estimation of proteolytic activity was carried out by measuring the liberation of free NH_2 groups from a gelatin substrate by the Van Slyke method. All results are expressed in terms of ml. 0.1 *N* base equiv. NH_2 -groups liberated. Unless otherwise stated, the enzyme studies were made with cell-free solutions obtained by centrifuging and filtering the cultures through Seitz filters. Bacterial counts were made as previously described.

Secretion of proteinase

The relationship between bacterial growth and proteolytic activity is shown in Fig. 1. A horse meat broth medium (National Drug Co.) was inoculated with *Cl. histolyticum* and incubated at 37.5°. Samples were removed at intervals,

Fig. 1. Relation between growth and proteinase secretion of *Cl. histolyticum*. Abscissa: hr. bacterial growth after inoculation. Ordinate (left side): proteinase activity in ml. 0.1 *N* KOH. Ordinate (right side): no. of bacteria in 1 ml. $\times 10^{-5}$. □ = no. of bacteria. ○ = full activity of *Cl. histolyticum* proteinase. × = initial activity of *Cl. histolyticum* proteinase.

filtered through Seitz filters and both initial and full activities measured. Bacterial counts were made at the same time. Both initial and full activities of the proteinase in the culture filtrates increased with increase in bacterial count up to a maximum at about 24 hr. After this a sharp drop occurred in the number of bacteria present and the initial and full proteolytic activities also decreased, but

at a slower rate. These results indicated the improbability of the proteinase being liberated into the medium as a result of bacterial autolysis. If such were the case an increase in the proteinase activity would be expected to occur after the 24 hr. maximum. Such an increase has never been observed. The proteinase which is activated by $\text{Fe}^{++}\text{-SH}$ is already present in the filtrates 6 hr. after the inoculation, and increases rapidly with increase in bacterial count. This is doubtless an example of true bacterial enzyme secretion. In contrast to this finding, Maschmann claims that just this particular proteinase which can be activated by $\text{Fe}^{++}\text{-SH}$ is the "intracellular proteinase, which is liberated only by autolytic processes occurring after the death of the bacteria, and which makes its appearance in the culture medium at a relatively late stage of incubation".

When the bacteria are separated by centrifuging and Seitz filtration from a culture which has not yet reached its maximal growth (15 hr.), it is found that the cell-free filtrate contains practically the whole of the proteolytic activity. The bacteria themselves attack gelatin only slightly.

Example: a 100 ml. portion of 3% neopeptone, pH 7.4, was inoculated with *Cl. histolyticum*, and after 15 hr. incubation at 37.5° was passed through a Seitz filter. One ml. of the bacteria-free filtrate gave, in 20 hr. incubation with gelatin under the usual conditions, an initial activity of 1.56 and a full activity of 2.62 (ml. 0.1 N alkali). The entire mass of bacteria removed by centrifuging and filtration was washed, suspended in saline and subjected to the action of a supersonic oscillator until cell destruction was about 80% complete. The proteolytic activity of the whole mass of broken-up bacteria, measured against gelatin as above was: initial, 0.26; full, 0.68. The proteolytic activity of the bacterial mass was only about 0.3% of that of the whole filtrate. That the bacteria themselves do show a slight proteolytic activity may be due to the fact that they still contain small amounts of preformed, unsecreted proteinase. Since in a 15 hr. culture rapid cell proliferation is still taking place and little autolysis has occurred, the fact that almost the entire amount of proteinase is found in the filtrate is further evidence that a true secretion of enzyme as expressed both by initial and full activities has occurred. The bacteria themselves contain a polypeptidase and a dipeptidase which pass into the culture medium only on autolysis and which are not secreted by young cells under conditions in which the proteinase is secreted. This fact also supports the idea that the latter is liberated not by autolysis but by a truly secretory process.

In a second, similar experiment, 4 l. 2% neopeptone were inoculated with *Cl. histolyticum* and incubated at 37.5° for 20 hr. The bacteria were collected by centrifuging, washed thoroughly with physiological saline and finally suspended in 10 ml. saline. 1 ml. of this suspension gave an initial activity against gelatin of 0.98 ml., and a full activity of 1.21 ml. When the bacterial suspension was first treated with the supersonic oscillator until 80% cell destruction occurred, 0.2 ml. gave the following activities: initial 1.31 ml.; full, 3.29 ml. Also in this experiment the activation behaviour of the proteinase obtained from the bacteria themselves did not differ from that previously observed for the enzyme secreted into the culture medium.

If the changes occurring in the bacterial count and the proteolytic activity (both initial and full) in a *Cl. histolyticum* culture are determined from time to time one finds, as previously shown, that a maximum occurs at about 24 hr., after which a steady decrease takes place. If to such an incubation mixture in which the bacterial count has passed its maximum and is decreasing rapidly, one again adds fresh sterile culture medium, the bacterial count rises and reaches a new maximum at 24 hr. Simultaneously both the initial and full proteolytic

activities of the medium also increase to a new maximum. Further incubation beyond this point results again in a decrease in both bacterial count and proteolytic activity. A typical experiment is shown in Fig. 2. Enzyme activities were determined as before, on cell-free Seitz filtrates of the culture medium.

Fig. 2. Relation between bacterial growth and proteinase secretion. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. 0.1 N KOH. Ordinate (right side): number of bacteria in 1 ml. $\times 10^{-8}$. $\square$ = no. of bacteria. $\circ$ = full activity of *Cl. histolyticum* proteinase. $\times$ = initial activity of *Cl. histolyticum* proteinase. Addition of new culture medium on the 7th day of bacterial growth.

Hydrolysis of clupein by the proteinase, and its activation by Fe^{++} -SH

When a bacteria-free culture filtrate of *Cl. histolyticum* (12 hr. growth) was allowed to act on gelatin or clupein, both substrates were split by the proteinase. In each case activation of the proteinase was obtained by addition of Fe^{++} -SH. Since in 12 hr. maximal bacterial growth has not nearly been reached (see Fig. 1) although clupein and gelatin are decomposed by the bacterial filtrate, this effect must be due to a proteinase which is secreted. After maximal growth has passed and autolysis of the bacteria occurs there is no increase in enzyme activity, either initial or full, towards clupein, as reported by Maschmann, but rather a decrease (Table I).

Table I. *Hydrolysis of clupein by Cl. histolyticum proteinase*

Cl. histolyticum grown in a solution of 3% heart infusion broth (Difco-standardized) at pH 7.4. After 12, 24 and 48 hr. growth at 37.5° samples were taken out and the Seitz-filtered enzyme solutions used for the exp. Determination: 2 ml. enzyme solution, 1 ml. cysteine-HCl (10 mg., neutralized), 0.6 ml. $M/10$ $FeSO_4$, or 1.6 ml. water, 2 ml. citrate-phosphate buffer pH 7.0 (McIlvaine) and 2 ml. 6.6% gelatin (neutralized) or 2 ml. of clupein sulphate (=50 mg. adjusted to pH 7.0). Digestion time was 5 hr. at 37.5°.

Age of bacterial culture (hr.)	Activity of the proteinase			
	Gelatin substrate		Clupein substrate	
	Initial	Full	Initial	Full
12	0.28	0.51	0.26	0.39
24	0.57	1.14	0.38	0.73
48	0.40	0.85	0.27	0.55

Since the activation behaviour, as well as the secretion, was found to be practically identical with respect to clupein and gelatin hydrolysis, there seems to be no reason to assume that the clupein-splitting enzyme differs from the one attacking gelatin, as Maschmann claims.

Stability of the proteinase

The decrease in both the initial and the full proteolytic activities after maximal bacterial growth has occurred is surprising. This decrease has been studied in three different culture media. It was found that the highest stability is shown in a casein medium. In neopeptone the stability is less, and about equal to that in horse meat broth (see Figs. 3 and 4). It seemed likely that the decrease

Fig. 3.

Fig. 4.

Fig. 3. Stability of *Cl. histolyticum* proteinase on neopeptone as culture medium. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. 0.1 *N* KOH. Ordinate (right side): number of bacteria in 1 ml. $\times 10^{-5}$. $\square$ = no. of bacteria. $\circ$ = full activity of *Cl. histolyticum* proteinase. $\times$ = initial activity of *Cl. histolyticum* proteinase.

Fig. 4. Stability of *Cl. histolyticum* proteinase on casein as culture medium. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. 0.1 *N* KOH. Ordinate (right side): no. of bacteria in 1 ml. $\times 10^{-5}$. $\square$ = no. of bacteria. $\circ$ = full activity of *Cl. histolyticum* proteinase. $\times$ = initial activity of *Cl. histolyticum* proteinase.

in the activity of the secreted proteinase in the culture medium might be due to inactivation during the long periods of incubation at 37.5°. Table II shows that this is not the case, since cell-free filtrates kept at 37.5° retain their proteolytic activity much longer than do unfiltered portions of the same incubation mixture. It is probable that the decrease is related to some process occurring during the autolysis of the bacteria.

Table II. *Stability of Cl. histolyticum proteinase*

Horse meat broth medium, 24 hr. culture; one portion (A) of the culture was allowed to stand at 37.5° without previous removal of the bacteria; a second portion (B) was freed from bacteria by Seitz filtration and also incubated at 37.5°; a third portion (C) was freed from bacteria and allowed to stand in the ice box. Enzyme determinations were made every 24 hr.

Days of incubation at 37.5°	Proteinase activity					
	A		B		C	
	Initial	Full	Initial	Full	Initial	Full
0	1.24	2.14	1.24	2.14	1.24	2.14
1	0.58	1.42	1.20	2.88	1.35	2.40
2	0.40	1.17	1.20	2.22	1.32	2.38
3	0.32	0.97	1.20	2.18	1.36	2.41
4	0.22	0.76	0.97	2.12	1.37	2.42

SUMMARY

The proteinase of *Cl. histolyticum* is liberated into the culture medium by secretion from the living bacteria, and is activated by Fe^{++} -cysteine under all conditions. On the basis of its activation behaviour, and its effect on various protein substrates, it appears to be identical with the proteinase obtained by breakdown of the bacterial cell.

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