183. THE PROTEINASES OF *CLOSTRIDIUM HISTOLYTICUM*

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THERE is some disagreement in the literature concerning the proteinases of *Cl. histolyticum*. Kocholaty *et al.* [1938] have claimed that this organism secretes only one proteinase into the medium, this proteinase being extracellular and strongly activated by cysteine. Maschmann [1938], on the other hand, has found results indicating that *Cl. histolyticum* secretes one or more extracellular proteinases which are not activated by cysteine, and that only during later stages of growth of the culture is a clupein-splitting enzyme secreted which is activated by cysteine, and which is presumably intracellular. Maschmann ascribes the cysteine activation of the filtrates of Kocholaty *et al.* to the presence of this clupein-splitting enzyme, but Kocholaty *et al.* have shown that their filtrates are strongly activated by cysteine from the time of the first appearance of the proteinase.

In the work here reported results were found which probably explain this disagreement. It was observed that a strain of *Cl. histolyticum* at first produced filtrates the properties of which were similar to those of Kocholaty *et al.* After repeated sub-cultivation of the strain on cooked meat cultures, in a tryptic digest of casein the organism began to secrete into the medium a system of proteinases, the properties of which were similar to those described by Maschmann.

Methods

Bacterial filtrates. The medium used was the modification of the tryptic digest of casein of Cole & Onslow [1916] as described by Gladstone & Fildes [1940]. In this medium the addition of yeast extract, cysteine or 2·5 % Difco proteose peptone did not bring about an increase in the yield of proteinase. 15 ml. inoculations of 24 hr. cultures of *Cl. histolyticum* (Strain 503, National Collection of Type Cultures) in meat broth tubes were made into 2·5 l. quantities of the medium in 3 l. round-bottomed flasks. After inoculation the flasks were evacuated to 30 mm. Hg, filled with H_2 and incubated at 37° for 16 hr. Cultures grown in this way produced as much proteinase as cultures incubated in a Fildes and McIntosh anaerobic jar. After incubation the cultures, *p*H *c.* 7·2, were clarified by adding about 15 g. kieselguhr per l. and filtering through Whatman no. 1 filter paper with suction. This filtration, which occupies only a few minutes, gave completely clear filtrates the proteinase activity of which was the same as those filtered through Seitz filters.

Estimation of proteinase activity. Since it was necessary to measure true proteinase activity, as distinct from proteolytic activity, and since filtrates of *Cl. histolyticum* cultures are known to contain poly- and di-peptidases, methods which involve the estimation of increase in free amino groups were excluded. In the first stages of this work the following method for estimating proteinase activity was used: 2 ml. of a neutral solution of Hammersten's casein containing 75 mg. casein per ml.; 2 ml. of McIlvaine's buffer, *p*H 7·0; 2 ml. of water, or of a solution of activator or inhibitor; and 2 ml. of the enzyme solution were pipetted into 12 ml. tared centrifuge tubes. The mixtures were then incubated at 37° for 30 min., after which the undigested casein was precipitated by the

addition of 2 ml. 30 % trichloroacetic acid. The precipitate was then centrifuged, resuspended in 10 ml. of 0·5 % acetic acid, and again spun down. After being dried for 1 hr. at 115° the tubes were weighed. The decrease in weight of the casein precipitate, as compared with a control with boiled enzyme, gave a measure of the amount of casein digested by the enzyme. Although some results obtained by this method are recorded, it was later abandoned because of the difficulty experienced in drying the precipitates to constant weight. A more satisfactory method was found to consist in the estimation of non-protein-N formed during digestion. 4 ml. of a 2·5 % solution of casein + 1 ml. of water, or of a solution of activator or inhibitor, were warmed to 40° in a boiling tube and then 1 ml. of the enzyme solution was added. After 15 min. incubation at 40° the undigested protein was precipitated by the addition of 10 ml. of 0·3 *M* trichloroacetic acid and filtered off. N was determined in aliquots of the filtrates by the micro-Kjeldahl method. Enzyme-N was determined by micro-Kjeldahl estimations on aliquots of the dialysed enzyme solutions.

Since these (and all other) methods for estimating proteinase activity cannot be specific for a particular proteinase, and therefore measure *total* proteinase activity, the term "the proteinase" should be read as "the proteinase or proteinases" throughout this paper.

Results

When this work was first started the *Cl. histolyticum* culture filtrates invariably contained a proteinase, the activity of which was increased by about 50 % by the addition of cysteine. This cysteine activation was quantitatively the

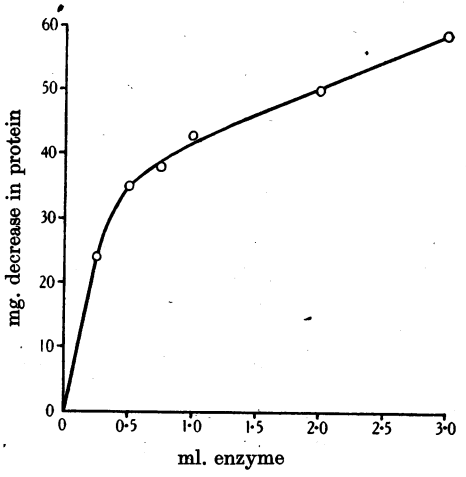

Fig. 1. Activity curve for crude filtrate. 150 mg. substrate, 3 hr. digestion.

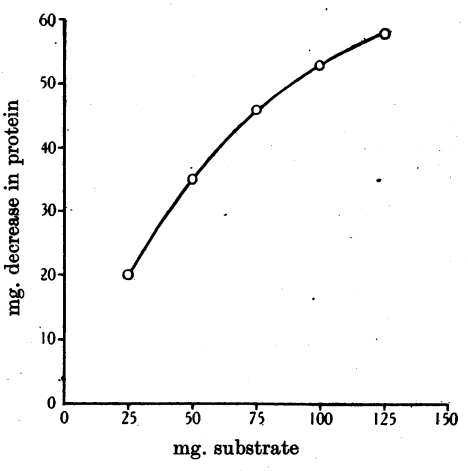

Fig. 2. Influence of substrate concentration on digestion by crude filtrate. 2 ml. filtrate, 3 hr. digestion.

same, whether the cultures were filtered after 8, 12 or 16 hr. incubation. The activation could be further increased in the presence of *M*/150 $FeSO_4$. Typical figures for the decrease in trichloroacetic acid-precipitable casein after 4 hr. digestion by the crude filtrates are:

2 ml. filtrate alone	43 mg.
2 ml. filtrate, *M*/150 cysteine	69 mg.
2 ml. filtrate, *M*/150 cysteine, *M*/150 $FeSO_4$ ...	87 mg.

These findings are in agreement with those of Kocholaty *et al.*

The activity curve and a curve showing the effect of substrate concentration for the crude filtrate are given in Figs. 1 and 2.

The filtrates obtained with the medium used contained very little protein and consequently it was impossible to obtain quantitative recovery of the proteinase by fully saturating the filtrates with ammonium sulphate. It was found, however, that the filtrates could be concentrated tenfold by distillation at 15 mm. pressure and about 15° internal temperature without appreciable loss of activity. From dialysed concentrates prepared in this way the proteinase could be precipitated by the addition of two volumes of methyl alcohol and thus recovered with yields of about 90 %. The precipitates could be dried, but with a loss of about 40 % of their activity. The dried powders retained their activity, without loss, for at least 3 months. The specific activity of the proteinase (mg. protein decrease per mg. enzyme N in 30 min.) was increased about fourfold by precipitation with methyl alcohol, and the activity of the precipitates was increased by cysteine to the same extent as the crude filtrates, as can be seen from the following typical figures:

	Specific activity	
	Without cysteine	With cysteine
Crude filtrate	65	91
Methyl alcohol precipitate	273	370
Dried precipitate	164	230

Fig. 3 shows the time curves for the partially purified proteinase. It will be seen that the increase in free NH_2-groups continues after the decrease in protein has stopped. This might be due to further action of the proteinase on the partially split protein, or to the action of poly- and di-peptidases.

Attempts to increase the specific activity of the proteinase by further fractional precipitation with organic solvents or with ammonium sulphate were not successful. It was found that the proteinase could be quantitatively adsorbed from a solution containing the methyl alcohol powder (10 mg./ml.) by kaolin (4 mg./ml.) at *p*H 5. A number of eluents was tried, and borate buffer at *p*H 9 was found to be the most effective. With this eluent a preparation was made which was inactive unless cysteine was added. Not more than about 30 % of the original activity could be recovered by this process of adsorption and elution. Furthermore, the proteinase could not be concentrated by the elution since it was found that when the volume of the eluent was smaller than the volume from which the proteinase had been adsorbed the recovery was correspondingly lower; nor was it possible to concentrate the enzyme by adsorbing from a more concentrated solution. The specific activity of the eluted proteinase (mg. protein decrease per mg. enzyme N in 30 min.) was 835, a ninefold increase with respect to the cysteine-activated crude filtrate. The method of adsorption and elution was not suitable as a preparative procedure, and further purification of the eluted proteinase was not possible. Its chief interest lies in the fact that the preparation was entirely inactive in the absence of cysteine. It was also found that $M/100$ cyanide could activate this preparation. Dialysis of the system proteinase-reducing agent caused the activity to disappear, but it could be restored by the addition of cyanide or cysteine.

Variation in the strain. After the strain of *Cl. histolyticum* had been maintained for about 3 months by weekly sub-cultivation from meat tubes the cultures of the organism began to show diminishing proteinase activity, without apparent decrease in bacterial growth. Within 2 weeks of this diminution being

observed the cultures acquired a very strong odour and the filtrates became completely inactive. Examination showed that the cultures were not contaminated, but it was noticed that tube cultures of the organism grew equally well aerobically and anaerobically. The organism was plated out on broth-agar and incubated for 48 hr. in an anaerobic jar. After incubation 6 colonies were picked off and each was sown into a separate meat tube. After 48 hr. incubation in the anaerobic jar inoculations from each of the meat tubes were made into two series of broth tubes. One of these series was incubated anaerobically, and the other aerobically, for 48 hr. At the end of incubation it was observed that

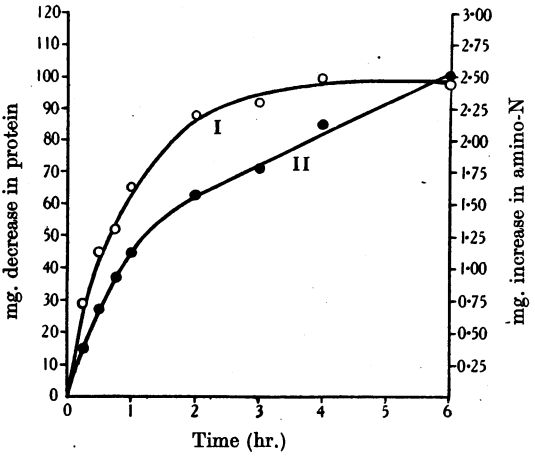

Fig. 3. Time curves for partially purified preparation. I, decrease in protein precipitable by trichloroacetic acid. II, increase in free NH_2-groups (Van Slyke manometric method, 5 min. with HNO_2).

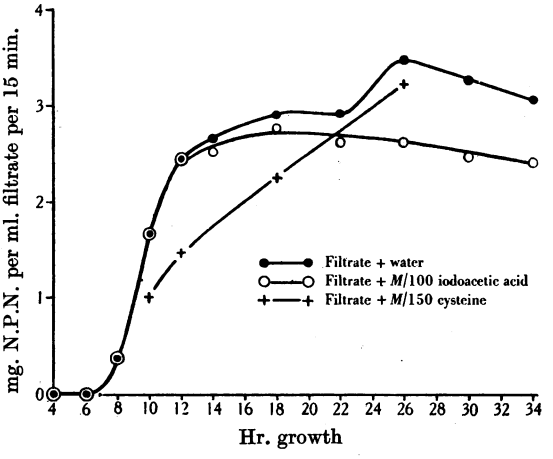

Fig. 4. Production of extra- and intra-cellular proteinases during growth of culture.

there was good growth in all the anaerobic tubes and in three of the aerobic tubes. The other aerobic tubes showed no growth. One of the cultures which grew anaerobically, but not aerobically, was then chosen for further cultivation. Inoculations were made from the appropriate meat tube into 12 tubes containing alkaline egg medium, which were then sealed and stored. When in the later stages of the work it was found that the activity of the cultures was diminishing the organism was recultivated from the spores in one of these tubes. When these new cultures were inoculated into 2·5 l. quantities of the tryptic digest of casein a good, odourless growth was obtained after 16 hr. incubation. The activity of the filtrates was now about 50% greater than the activity of the previous filtrates, but, on the other hand, this activity could not be increased by the addition of cysteine or cyanide. The relation between the new and old cultures can be seen from the following table:

	mg. protein decrease per 2 ml. filtrate in 30 min.		
	Without cysteine	With cysteine	Odour
Old cultures	20	30	Slight
Inactive cultures	0	0	Very strong
New cultures	30	30	None

With the new filtrates it was again possible to prepare methyl alcohol precipitates from dialysed concentrates, and the proteinase could be quantitatively adsorbed by kaolin from solutions of these precipitates. Borate buffer, *p*H 9, again proved to be the most effective eluent, and the recovery of activity was again about 30%. The proteinase in the eluates had a very low but definite activity in the absence of cysteine, but in the presence of cysteine the activity was increased fivefold. All proteinase estimations given below were done by determining the increase in non-protein-N. The specific activity (mg. non-protein-N formed per mg. enzyme N in 15 min.) of the cysteine-activated eluates was 580. The proteinase could not be concentrated in the eluates, and consequently a further increase in the specific activity of the proteinase could not be effected. When the eluate + *M*/150 cysteine was incubated with *M*/100 iodoacetic acid the activity was reduced to one-third, but never quite to the level of the eluate alone. It was then found that the activity of the methyl alcohol precipitates was reduced by about 30% by *M*/100 iodoacetic acid. The amount of activity in the borate eluates which was due to cysteine corresponded to the loss of activity in the methyl alcohol precipitates due to iodoacetic acid. Typical figures for equivalent amounts of enzyme are given below:

Enzyme preparation	mg. N.P.N. formed in 15 min.
Methyl alcohol precipitate	3·07
Do + *M*/150 cysteine	3·00
Do + *M*/100 iodoacetic acid	2·18
Borate eluate	0·25
Do + *M*/150 cysteine	1·36
Do + *M*/150 cysteine + *M*/100 iodoacetic acid	0·43

These figures indicate that the methyl alcohol precipitate consisted of at least two proteinases, of which one was not activated by cysteine and not inhibited by iodoacetic acid, and the other was activated by cysteine and inhibited by iodoacetic acid. It appeared that both these enzymes were adsorbed by kaolin, and that one of them was quantitatively and nearly specifically eluted by borate buffer at *p*H 9.

In order to follow the secretion of these enzymes during the growth of the culture the following experiment was carried out: 1·5 l. of the inoculated medium were placed in a 2 l. flask which was kept at 37°. Sterile H_2 was bubbled through the medium and at regular intervals 10 ml. samples of the culture were withdrawn under sterile conditions. The samples were filtered through Seitz filters and aliquots were incubated with water, *M*/100 iodoacetic acid and *M*/150 cysteine respectively for 30 min. before being tested for proteinase activity. The results are plotted in Fig. 4. It will be seen that up to the 12th hr. of growth the proteinase which appeared in the medium was neither inhibited by iodoacetic acid nor activated by cysteine; it was, in fact, quite strongly inhibited by cysteine. After 12 hr. another proteinase appeared in the medium. This proteinase was inhibited by iodoacetic acid and activated by cysteine, and was presumably an intracellular enzyme. These findings are in agreement with those of Maschmann [1938]. The fact that a 16 hr. filtrate contained one set of enzymes that was inhibited by cysteine, and another that was activated by cysteine, explains why cysteine had no apparent effect on the crude filtrates.

The intracellular proteinase could be separated to a fair degree from the mixture of intra- and extra-cellular proteinases by adsorption on kaolin, followed by elution with borate buffer, as already described. The extracellular proteinase could be obtained free from the intracellular proteinase by filtering the culture after 8 hr. growth, i.e. before the appearance of the intracellular proteinase in

the medium. The specific activity of the extracellular proteinase in the crude 8 hr. filtrates was 19·4 (mg. non-protein-N formed per mg. enzyme-N in 15 min.). After concentration, precipitation with fully saturated ammonium sulphate and precipitation with 66 % methyl alcohol a preparation was obtained with a specific activity of 86, but further attempts at purification were unsuccessful. This preparation was unaffected by iodoacetic acid and inhibited about 40 % by cysteine.

Discussion

These results show that strains of *Cl. histolyticum* on continued sub-cultivation vary in properties when picked off as a single colony. The proportion of proteolytic enzymes is only one among the properties which vary in this way; and this provides an explanation for the apparent contradictions of previous workers in this field.

Summary

It was found that a strain of *Cl. histolyticum* produced an extracellular proteinase which was activated by cysteine. After continued sub-cultivation the strain was plated out and cultures from a single colony produced an extracellular proteinase which was inhibited by cysteine and an intracellular proteinase which was activated by cysteine.

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