CVIII. STUDIES ON PROTEINASES OF SOME ANAEROBIC AND AEROBIC MICRO-ORGANISMS

By LEOPOLD WEIL, WALTER KOCHOLATY
AND LOUIS DeSPAIN SMITH

From the Biochemical Research Foundation of the Franklin Institute, Philadelphia, Pa.

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Clostridium histolyticum secretes a proteinase, with a *p*H optimum of 7, which can be activated markedly by —SH compounds; and this activation can be increased by the addition of traces of heavy metal [Weil & Kocholaty, 1937; Kocholaty, Weil & Smith, 1938]. The results of the present study, an investigation of the proteinases of several other Clostridia, indicate that the proteinases of some other members of this genus have similar *p*H optima and activation characteristics.

Cell-free filtrates of *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacillus mycoides* and *Staphylococcus citreus* contain proteinases which, although differing in their partial activation behaviour (with cysteine alone and Fe^{++} alone), as a whole resemble the proteinases of the members of the *Clostridium* group.

Experimental

The bacterial proteinase solutions were prepared in the same manner as those previously reported [Weil & Kocholaty, 1937] by inoculating the bacteria into culture media and filtering through a Seitz filter after 18–24 hr. anaerobic incubation at 37°. For *Cl. histolyticum* and *Cl. Welchii*, horse meat broth was used; for *Cl. sporogenes*, a 3 % solution of Difco neopeptone; for *Cl. putrificum* and *Cl. botulinum*, beef heart infusion broth. The *p*H of the culture media was adjusted to about 7·4. The organisms used were obtained from the American Type Culture Collection. The strain of *Cl. histolyticum* was in the rough phase [Hoogerheide, 1937].

One ml. of *Cl. histolyticum*, 2 ml. of *Cl. Welchii* and 3 ml. of *Cl. sporogenes* or *Cl. botulinum* filtrates, respectively, were taken for each determination. To this amount of bacteria-free filtrate, either 10 mg. of cysteine-HCl (neutralized), 0·6 ml. of 0·1 *N* $FeSO_4$, or both, were added. After the addition of 3 ml. of citrate-phosphate buffer at the proper *p*H, 3 ml. of a 3 % gelatin solution of the same *p*H were pipetted into the reaction mixture. The total volume was made up to 10 ml. After an incubation of 22 hr. in a hydrogen atmosphere at 37°, the increase of amino groups was measured by the Van Slyke method, and the results were expressed in ml. of 0·05 *N* KOH. All the filtrates of the Clostridia were practically free of peptidases, and the activities measured in this way were attributable to the proteinase action alone.

From Table I it can be seen that the proteinases of the Clostridia resemble very much, with respect to *p*H optima and activations, the proteinase of *Cl. histolyticum*, already described [Weil & Kocholaty, 1937].

Maschmann [1938, 1] stated that the cell-free filtrate of *Cl. Welchii* without activation could hydrolyse only gelatin, although, when activated by —SH compounds, clupein was hydrolysed. The other protein substrates that he

Table I. *Activation and pH optima of the proteinases of some Clostridia measured on gelatin*

	*p*H...	4	5	6	7	8
	Activator		Activity in ml. of 0·05 *N* KOH			
Cl. histolyticum	None	0·0	0·56	3·52	4·32	3·62
	Cysteine	0·0	1·20	4·84	5·62	4·98
	Fe^{++}	0·0	0·52	3·20	4·00	3·08
	Cysteine-Fe^{++}	0·10	1·74	5·82	7·16	5·64
C. Welchii	None	0·04	—	0·94	1·20	1·00
	Cysteine	0·20	—	1·96	2·24	2·04
	Fe^{++}	0·24	—	3·22	3·70	3·12
	Cysteine-Fe^{++}	0·24	—	3·00	3·28	2·74
Cl. sporogenes	None	0·11	—	2·10	2·44	2·01
	Cysteine	0·14	—	3·06	3·58	3·12
	Fe^{++}	0·22	—	4·01	4·22	3·82
	Cysteine-Fe^{++}	0·30	—	5·81	6·46	5·76
Cl. putrificum	None	0·00	—	1·37	1·65	1·01
	Cysteine	0·09	—	1·72	2·04	1·29
	Fe^{++}	0·32	—	2·41	2·81	1·68
	Cysteine-Fe^{++}	0·22	—	2·16	2·36	1·64
Cl. botulinum	None	0·08	—	0·49	0·77	0·56
	Cysteine	0·17	—	0·67	0·97	0·76
	Fe^{++}	0·30	—	0·83	1·18	0·98
	Cysteine-Fe^{++}	0·24	—	0·81	1·16	0·99

investigated (ovalbumin, casein, Witte peptone) were but slightly hydrolysed. The proteinase responsible for gelatin hydrolysis was found to be secreted into the medium in the early stages of bacterial growth. However, the proteinase which induces the hydrolysis of clupein was described as appearing only late in incubation, presumably after autolysis of the bacteria had occurred. From this, Maschmann assumed that this latter proteinase was an intracellular one. Similar conclusions were reached [Maschmann, 1938, 2] in connexion with the proteinases of *Cl. histolyticum* and *Cl. botulinum*.

Because of this description of an intracellular proteinase which promotes the hydrolysis of clupein, and of our previous negative results in regard to this point [Weil & Kocholaty, 1937; Kocholaty, Weil & Smith, 1938], careful attention has been given, in the present study, to factors affecting autolysis.

A cell-free filtrate of *Cl. Welchii*, obtained from a 16 hr. culture, was used as a source of proteinase. At this time, 16 hr. after inoculation, practically all of the bacteria were living and dividing, and consequently, any enzyme present in the culture filtrate may be presumed to have been produced extracellularly. Two ml. of the *Cl. Welchii* filtrate were used for each enzyme activity determination. The activations were carried out as described above. After the addition of 3 ml. of citrate-phosphate buffer at *p*H 7·0, 3 ml. of a 5 % solution of gelatin, clupein sulphate, Witte peptone or casein at *p*H 7·0 were added. After 20 hr. of anaerobic incubation at 37°, the increase in amino groups was measured by the Van Slyke method. In the case of casein, alcoholic titration was used and the buffer was omitted, because of its disturbing effect on the titration. The *p*H, however, was adjusted in this special case.

As Table II shows, the proteinase of *Cl. Welchii* readily hydrolysed all the investigated proteins, and in every case a marked activation was obtained with cysteine-Fe^{++}. The similar behaviour of the enzyme-activator system toward the investigated protein substrates did not indicate a number of proteinase actions. This enzymic picture resembles very much that obtained in our previous work [Weil & Kocholaty, 1937] on the proteinase of *Cl. histolyticum*.

Table II. *The decomposition of various protein substrates by* Cl. Welchii *proteinase*

	Activator			
	None	Cysteine	Fe^{++}	Cysteine-Fe^{++}
Substrate	Activity in ml. of 0·05 N KOH			
Gelatin	0·84	1·91	1·01	1·91
Clupein	0·57	1·65	0·77	2·19
Witte peptone	0·60	0·00	0·10	1·40
Casein	0·56	1·28	0·76	1·32

A recent paper by Berger *et al.* [1938] also indicates a possible importance of reducing substances for the peptidase system of anaerobic bacteria. A similar observation was reported by Maschmann [1938, 3], and he made the suggestion that the activation observed by us on the proteinase of *Cl. histolyticum* was due to the peptidase system. However, such an explanation does not hold for the proteinases of Clostridia. Bacteria-free filtrates of the *Clostridium* group after 1 day's cultivation were found to contain practically no peptidase, whether tested for in the presence or absence of the activator system. The peptidases, as has been shown [Kocholaty, Smith & Weil, 1938], are intracellular, and appear in the bacterial filtrate only after the autolysis of the bacteria.

Further evidence that the activation is due to the proteinase action was given by the liquefaction of gelatin by the proteinase of *Cl. histolyticum*. The

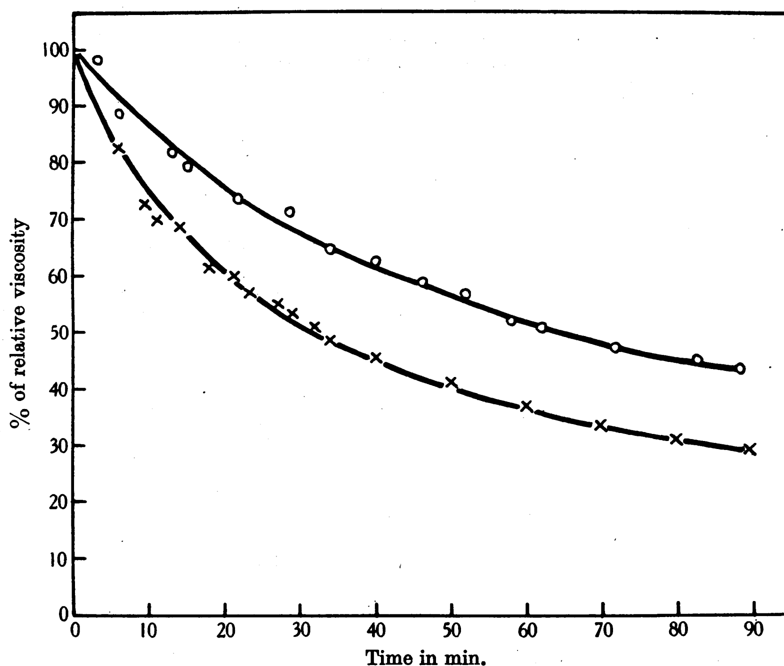

Fig. 1. o—o Without activator. × — × With activator.

hydrolysis of gelatin by *Cl. histolyticum* proteinase, measured by the viscosimetric method, was markedly activated by cysteine-Fe^{++}. This method of proteinase determination measures the very first step of protein breakdown, one

which is indeterminable by any chemical method. The activation of this first step of hydrolysis cannot, therefore, be explained by a peptidase action. One ml. of *Cl. histolyticum* filtrate, diluted 1:80, and 3 ml. of 3 % gelatin solution at *p*H 7·0 were used, the total volume being 4 ml. The activation was carried out as usual. The drop in the viscosity was measured at 37° and is expressed in percentage of the original viscosity (Fig. 1).

Characteristics of the proteinases of some aerobic bacteria

For the preparation of proteinase of *B. mycoides*, a pure culture was inoculated into a medium containing 0·3 % beef extract, and 0·5 % yeast extract. After an aerobic incubation of 5 days at 37°, the culture was filtered. Bacteria-free proteinase solutions of *Staph. citreus*, *Ps. fluorescens*, *S. marcescens* and *Proteus vulgaris* were obtained through inoculation of the bacteria into heart-infusion broth. After 48 hr. aerobic incubation at 37°, the cultures were filtered. For each enzyme determination 3 ml. of the filtrate were used, and the estimations were carried out in the same way as those described for the proteinases of members of the *Clostridium* group.

Table III, which represents the activations and *p*H-optima of proteinases derived from *B. mycoides*, *Staph. citreus*, *Ps. fluorescens*, and *S. marcescens*, presents a uniform picture. The addition of cysteine, which activated the proteinases of the *Clostridium* group, produced in these cases a partial inhibition, while Fe^{++} had an activating effect. Cysteine-Fe^{++} gave the maximal activation. The mechanism of these activations, however, requires further investigation. The *p*H-optima of the investigated proteinases were at neutrality, which is in agreement with the results of Gorbach & Pirch [1936] and Maschmann [1937], who worked on the proteinase of *Ps. fluorescens*.

Table III. *Activation and* p*H-optimum of the proteinases of some aerobic bacteria, measured on gelatin*

	*p*H...	4	6	7	8
	Activator	Activity in ml. of 0·05 *N* KOH			
B. mycoides	None	0·00	0·24	0·48	0·36
	Cysteine	0·00	0·12	0·28	0·14
	Fe^{++}	0·00	0·40	0·65	0·49
	Cysteine-Fe^{++}	0·00	0·48	0·75	0·54
Staph. citreus	None	0·00	0·78	1·12	0·64
	Cysteine	0·00	0·64	1·06	0·54
	Fe^{++}	0·20	2·32	2·60	2·24
	Cysteine-Fe^{++}	0·28	1·61	3·40	2·46
Ps. fluorescens	None	0·00	0·40	0·68	0·46
	Cysteine	0·00	0·16	0·37	0·25
	Fe^{++}	0·11	0·80	1·21	0·78
	Cysteine-Fe^{++}	0·10	0·88	1·23	0·86
S. marcescens	None	0·00	0·91	1·61	1·36
	Cysteine	0·00	0·64	1·16	0·48
	Fe^{++}	0·00	1·61	2·23	1·82
	Cysteine-Fe^{++}	0·08	1·69	2·52	2·08

The results given in Table IV, on the proteinase of *Pr. vulgaris*, resemble very much those obtained with the proteinases of the Clostridia. The difference between the activation behaviour of the proteinase of *Pr. vulgaris* and the proteinases of the aerobic organisms may be due to the fact that *Pr. vulgaris* is a facultative micro-organism. It is, however, quite possible that, by cultivating a facultative micro-organism for many generations aerobically, a proteinase may

Table IV. *Activation and* p*H-optimum of the proteinase of* Pr. vulgaris

Activator	*p*H... 4	6	7	8
	Activity of ml. of 0·05N KOH			
None	0·00	0·41	0·75	0·48
Cysteine	0·00	0·40	0·86	0·49
Fe^{++}	0·00	0·37	0·68	0·40
Cysteine-Fe^{++}	0·00	0·99	1·56	1·13

be obtained which would have the characteristics of those obtained from the aerobic group of organisms. The possibility of such variability was demonstrated previously [Kocholaty & Weil, 1938].

Summary

Anaerobic organisms, including *Cl. histolyticum*, *Cl. sporogenes*, *Cl. Welchii*, *Cl. putrificum* and *Cl. botulinum*, secreted proteinases which were activated by cysteine. The combination of cysteine with Fe^{++} gave the maximum activation.

The exocellular proteinase of *Cl. Welchii* hydrolysed clupein, gelatin, casein and Witte peptone.

Proteinases of certain aerobic organisms, *B. mycoides*, *Staph. citreus*, *Ps. fluorescens* and *S. marcescens*, were partially inhibited by cysteine. However, marked activations were effected by cysteine in combination with Fe^{++}.

The proteinase of the facultative anaerobe, *Pr. vulgaris*, resembled the proteinases of the Clostridia in activation behaviour.

Optimal action at neutrality was observed in all the investigated bacterial proteinases.

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