CCXIX. STUDIES ON THE ENDO-ENZYMES, PARTICULARLY THE PEPTIDASES, OF *CLOSTRIDIUM HISTOLYTICUM*

BY WALTER KOCHOLATY, LOUIS SMITH AND LEOPOLD WEIL

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

(Received 21 July 1938)

THE first noteworthy work on bacterial peptidases as endo-enzymes was that of Virtanen & Tarnanen [1931; 1932, 1, 2] on *B. fluorescens liquefaciens*. Later, Gorini *et al.* [1932] and Gorbach & Pirch [1936-7] reported on the same subject. These various authors agree that the bacterial peptidases are present exclusively within the bacterial cell, are not secreted and show optimal activity at an alkaline reaction. Also in no instance has it been possible to demonstrate the presence of a carboxypeptidase in the bacterial cell.

In all of the above-mentioned work use was made of toluene autolysis to liberate the peptidases from the intact cell. Investigations carried out in this laboratory on the strict anaerobe, *Cl. histolyticum*, have shown that this time-consuming method of autolysis can be dispensed with to advantage by use of a supersonic oscillator to disrupt the bacterial cells. With this apparatus it is possible to break up the bacterial cells in a very short time, which is of particular advantage in view of the extreme instability of the bacterial peptidases in solution. When the tissue debris is removed from such preparation by filtration, a cell-free, water-clear fluid containing considerable quantities of peptidases is obtained. In this way it was shown that young cultures of *Cl. histolyticum* contain a polypeptidase with pH optimum at about 8.7, and a dipeptidase with an optimum at pH 7.6. All attempts to detect a carboxypeptidase failed, and it appears fairly certain that such an enzyme does not exist in bacteria. The bacterial polypeptidase is activated to a considerable extent by Mg^{++} , a phenomenon previously described by Johnson *et al.* [1936] for the animal polypeptidase, leucylpeptidase. The bacterial dipeptidase appears to be indifferent toward Mg^{++} .

Among the deamidases, we were especially interested in the enzyme which deaminizes *l*-aspartic acid. The deamination of this amino-acid by suspensions of *Cl. histolyticum* occurs optimally at pH 7.6 with formation of succinic acid as previously shown by Cook & Woolf [1928]. The presence of an aspartase could not be demonstrated, although previously this enzyme had been found in suspensions of *B. coli commune* by Quastel & Woolf [1926], and in cell-free preparations of *B. fluorescens liquefaciens* by Virtanen & Tarnanen [1932, 1, 2]. We were however able to show the presence of other deamidases, which in a weakly alkaline medium attack glycine, *l*-leucine, *d*-alanine, *d*-glutamic acid and *l*-tryptophan with liberation of NH_3 .

In a previous publication Weil & Kocholaty [1937] suggested that the peptidases of *Cl. histolyticum* might be secreted by the intact cells. This idea was advanced because of the fact that no polypeptidase could be found in bacteria which had been autolysed with toluene. However, at that time determinations were made on very small quantities of bacteria and the activating effect of Mg^{++}

was unknown, so that the peptidase in question may have been present in the inactive form. The results now reported definitely indicate that these enzymes are not secreted, but must be classed as endo-enzymes.

EXPERIMENTAL

The micro-organism used, as in previous work, was the dissociated strain of *Cl. histolyticum* described by Hoogerheide [1937]. The culture medium used in all cases was a 2% solution of neopeptone (Difco standardized), adjusted before inoculation to pH 7.4. After an incubation period of 18–20 hr. at 40°, the bacteria were centrifuged down, washed carefully, resuspended in saline and used at once. Such a suspension contains a negligibly small amount of spores and cellular debris, most of the bacteria being intact. The pH of the suspensions was always 7.0. When studies were to be made on cell-free material the washed bacteria were disrupted in the supersonic oscillator, after which the cellular debris was removed by Seitz filtration. The enzyme solutions obtained in this way were extremely unstable.

Under the conditions employed, a 10 min. treatment of a bacterial suspension in the supersonic oscillator resulted in a destruction of about 50% of the cells. This could be increased to 80–85% by a further treatment of 10 min. In order to avoid destruction of the enzymes the disruption was never carried beyond that point. This method for preparing bacterial enzyme solutions is advantageous not only because the cellular disruption is accomplished rapidly but also because it avoids the use of toluene which is sometimes harmful to enzymes. However, because of the formation of slimy material, bacterial suspensions treated in this way are very difficult to filter.

The estimation of peptidase activity was carried out by measuring the increase in COOH or NH₂ groups produced in the substrates leucylglycine and leucylglycylglycine, using respectively the method of Foreman [1920], or of Van Slyke. The estimation of NH₃ was by the usual distillation method.

pH optimum of the peptidases

To 1 ml. portions of the above described cell-free extracts were added 3 ml. citrate-phosphate buffer (McIlvaine—for the higher pH values KOH was also added); 5 ml. *M*/5 leucylglycine or 5 ml. *M*/5 leucylglycylglycine, adjusted to the proper pH, were added as substrates. The mixtures were incubated for 7 hr. at 40°. pH estimations made before and after the incubation period showed practically no change. The increase in free NH₂ groups was measured by the Van Slyke method, the results being expressed in ml. of 0.1 *N* base (Figs. 1 and 2). It should be pointed out that in all experiments reported the polypeptidase was accompanied by dipeptidase, since no attempt was made to separate the two enzymes. The values for polypeptidase activity are therefore only approximations.

Activation of the polypeptidase by Mg⁺⁺

Reaction mixtures were prepared consisting of 1 ml. cell-free enzyme solution prepared as described above, 5 ml. *M*/5 leucylglycine or leucylglycylglycine previously adjusted to the optimal pH, 3 ml. of citrate-phosphate buffer at the optimal pH and 1 ml. of water or MgCl₂ solution, the last to make the final molarity in the reaction mixtures 0.005–0.020. The mixtures were incubated at 40° for 7 hr., the COOH increase being measured by the method of Foreman. Results (Fig. 3) are expressed in ml. of 0.1 *N* KOH.

Fig. 1.

Fig. 2.

Fig. 1. pH optimum of *Cl. histolyticum* dipeptidase. Abscissa: pH. Ordinate: dipeptidase activity in ml. 0.1 N KOH.

Fig. 2. pH optimum of *Cl. histolyticum* polypeptidase. Abscissa: pH. Ordinate: polypeptidase activity in ml. 0.1 N KOH.

Fig. 3 shows the effect of conc. of Mg^{++} on the activity of *Cl. histolyticum* polypeptidase. Table I shows some results obtained with the bacterial dipeptidase at pH 7.6, and with the polypeptidase at pH 8.7. The Mg concentration was 0.01 M in the final reaction mixture. Comparative determinations were made using both a suspension of intact cells, and a cell-free enzyme solution prepared by the supersonic method.

Table I. Effect of Mg^{++} on the activity of *Cl. histolyticum* peptidases

Enzyme source	Dipeptidase activity hr. incubation				Polypeptidase activity hr. incubation			
	1	4	7	24	1	4	7	24
Intact cells	—	—	—	0.91	—	—	—	1.24
Intact cells + Mg^{++}	—	—	—	0.90	—	—	—	2.00
Disrupted cells	0.55	1.18	2.23	—	1.24	2.54	3.41	—
Disrupted cells + Mg^{++}	—	1.15	—	—	2.04	—	3.76	—

Fig. 3.

Fig. 4.

Fig. 3. Activation of *Cl. histolyticum* polypeptidase by Mg^{++} . Abscissa: molarity of $MgCl_2$ added. Ordinate: polypeptidase activity in ml. 0.1 N KOH. $\times$: polypeptidase activity without Mg addition.

Fig. 4. pH optimum of *l*-aspartic acid deaminase (*Cl. histolyticum*). Abscissa: pH. Ordinate: ml. N/70 NH_3 liberated.

It is apparent that the bacterial dipeptidase is indifferent toward the presence of Mg^{++} . The extensive activation of the polypeptidase by Mg^{++} suggests that this enzyme might be largely inactivated by dialysis. Addition of Mg^{++} to such dialysed preparations should reactivate the enzyme, as was shown by Johnson *et al.* [1936] in the case of the intestinal leucylpeptidase. However, the polypeptidase preparations obtained were too unstable to test this point.

Cell-free Seitz filtrates, obtained from young actively-growing cultures (15 hr.) of *Cl. histolyticum* in which little autolysis has occurred, contain practically no dipeptidase or polypeptidase. Also from Table I it is seen that young washed intact suspensions of the bacteria show little peptidase action unless the incubations are carried out for long periods of time (24 hr.). The activity observed under such conditions is probably the result of enzyme liberation by autolysis, since toluene was present as a preservative during the long incubation period. But if the bacteria are previously broken up by the supersonic oscillator, high peptidase activity is observed even in short incubations. Such results indicate that these enzymes are endocellular, and are liberated only after destruction of the cell membrane by autolysis or other means.

Tests made under the same conditions for the presence of a bacterial carboxypeptidase, using chloroacetyl-*l*-tyrosine as substrate at *pH* 7.4 and 8.4, and with and without added Mg^{++} , were negative in each case.

Deamination of l-aspartic acid

For establishing the *pH* optimum of this enzyme, reaction mixtures were prepared consisting of 2 ml. bacterial suspensions (treated with the supersonic oscillator but not filtered), 2 ml. *M/10 l*-aspartic acid and 5 ml. citrate-phosphate buffer. The incubation period was 20 hr. at 40°. The results are shown in Fig. 4 in terms of ml. *N/70 NH*₃ formed. The *pH* optimum for the deamination was near 7.6.

Similar reaction mixtures, extending over the same *pH* range, but containing 2 ml. *M/10* fumaric acid and 10.7 mg. *NH*₄Cl in place of the *l*-aspartic acid (total volume, 11 ml.) gave no evidence of aspartic acid synthesis after 20 hr. incubation at 40°. No appreciable decrease in free *NH*₃ occurred, indicating that the enzyme aspartase was absent.

In another experiment a fairly strong disrupted bacterial suspension was allowed to stand at *pH* 7.6 for 36 hr. with 4 ml. *M/10* aspartic acid (40°). After filtration, 10% *H*₃*PO*₄ was added, and the solution extracted several times with ether. The ether extract was evaporated to dryness, taken up in water, and treated with animal charcoal. On evaporation a crystalline material, m.p. 185°, was obtained. A mixed m.p. with pure succinic acid showed no depression. For further identification, the Pb salt was prepared, using Pb acetate. This salt was obtained in its characteristic form, small whetstone-shaped crystals arranged in rosettes. The deamination of aspartic acid yields succinic acid, as previously reported by Cook & Woolf [1928].

Deamination of other amino-acids

Because of the scarcity of enzyme material, it was not possible to determine the *pH* optima of the deamidases which attack other amino-acids. However, it could be shown that enzyme solutions obtained by the disruption method from *Cl. histolyticum* do bring about aerobic deamination of glycine, *l*-leucine, *d*-alanine, *d*-glutamic acid and *l*-tryptophan at *pH* 7.8. To 3 ml. enzyme solution

were added 2 ml. *M*/10 amino-acid solution and 5 ml. citrate phosphate buffer (McIlvaine). After 48 hr. incubation at 40°, the NH_3 liberated was determined by the distillation method. The results are shown in Table II.

Table II. *Deamination of amino-acids by Cl. histolyticum enzymes*

Amino acid	ml. $\text{N}/70 \text{ NH}_3$ formed after 48 hr.
Glycine	4.34
<i>D</i> -Alanine	3.68
<i>L</i> -Leucine	4.86
<i>D</i> -Glutamic acid	5.70
<i>L</i> -Tryptophan	2.14

Intracellular nature of the enzymes

All attempts to show the presence of the above enzymes (dipeptidase, aminopolypeptidase, deaminases) in cell-free filtrates obtained from young rapidly growing cultures were unsuccessful. In agreement with other authors it must be concluded that these enzymes are not secreted during bacterial growth, but are liberated only upon autolysis of the cells.

SUMMARY

1. Solutions obtained by disrupting *Cl. histolyticum* suspensions with a supersonic oscillator contain a dipeptidase with a *pH* optimum of 7.6, and a polypeptidase acting optimally at *pH* 8.7.

2. The polypeptidase is activated by Mg^{++} , while the dipeptidase is unaffected.

3. The preparations contain also enzymes which attack various amino-acids with liberation of NH_3 .

4. All of the above enzymes are endo-enzymes, and are not secreted by the intact bacteria.

The authors wish to express their thanks to Dr Ellice McDonald, Director, for his interest and support throughout this work.

REFERENCES

- Cook & Woolf (1928). *Biochem. J.* **22**, 474.
 Foreman (1920). *Biochem. J.* **14**, 451.
 Gorbach & Pirch (1936-37). *Enzymologia*, **1**, 191.
 Gorini, Grassmann & Schleich (1932). *Hoppe-Seyl. Z.* **205**, 133.
 Hoogerheide (1937). *J. Bact.* **34**, 387.
 Johnson, Johnson & Petersen (1936). *J. biol. Chem.* **116**, 515.
 Quastel & Woolf (1926). *Biochem. J.* **20**, 545.
 Virtanen & Tarnanen (1931). *Naturwissenschaften*, **19**, 397.
 ——— (1932, 1). *Hoppe-Seyl. Z.* **204**, 247.
 ——— (1932, 2). *Biochem. Z.* **250**, 193.
 Weil & Kocholaty (1937). *Biochem. J.* **31**, 1255.