74. STUDIES ON DIFFUSING FACTORS

5. SEPARATION OF THE DIFFUSING FACTOR FROM THE PROTEOLYTIC ENZYMES PRESENT IN CULTURE FILTRATES OF *CLOSTRIDIUM WELCHII*

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(Received 29 February 1940)

INDIRECT evidence has recently been advanced [Madinaveitia, 1939] to show that the diffusing factor is not a proteolytic enzyme; since, under the experimental conditions used, testicular extracts did not hydrolyse gelatin it seemed almost certain that if diffusing factors from different sources are similar in their physiological action, their effects could not be due to proteolysis. A more conclusive proof is the separation of proteolytic and diffusing activities in culture filtrates of *Clostridium Welchii* which has now been accomplished. Despite the qualitative nature of the assay method for diffusing activity and its very large margin of error it was possible to demonstrate this separation in culture filtrates fractionated by various methods.

A dialysed *Cl. Welchii* filtrate adjusted to pH 4.0 deposits on standing a precipitate which contains most of the proteolytic activity of the starting material but is devoid of diffusing properties. Separation can also be effected by fractional adsorption on alumina C- γ or by fractional elution of kaolin or alumina adsorbates. It is evident from the results that the diffusing factor of *Cl. Welchii* is quite distinct from the gelatin-splitting enzymes produced by this organism.

Experimental

Culture filtrates of *Cl. Welchii* were concentrated *in vacuo* (23°) to 1/10 of their volume and dialysed for two days against running tap water. The dialysed solutions were concentrated *in vacuo* (23°) to the original volume of culture filtrate.

For estimation of the proteolytic activity of the different fractions 1 ml. of enzyme solution was incubated with 9 ml. 5% gelatin in *M*/15 phosphate buffer pH 7.0 containing 0.05% cysteine hydrochloride. The increase of carboxyl groups was estimated in 2 ml. samples after 15–24 hr. incubation at 38° by the method of Willstätter & Waldschmidt-Leitz [1921].

The diffusing activities of the different fractions were compared by the recently described modification [Madinaveitia, 1939] of the original method [Madinaveitia, 1938]. The figures given represent the average of double determinations in at least two rabbits.

Concentration of the proteolytic activity

To portions of 10 ml. of the dialysed filtrate 1 ml. of *M* acetate buffer of varying pH was added and each portion adsorbed with 0.5 g. kaolin (B.D.H. acid-washed). After 1 hr. the adsorbates were centrifuged off and the proteolytic activity of each supernatant estimated.

Table 1

Increase in ml. <i>M</i> /20 KOH	Original solution	Supernatants			
		pH 3	pH 4	pH 5	pH 6
	1.30	0.15	0.20	0.60	1.30

Further adsorptions were always carried out at pH 4.0 because of the danger of destroying the diffusing factor at a lower pH.

Different eluents were tried. 10 ml. lots of a dialysed culture filtrate adjusted to pH 4.0 were adsorbed with 1 g. of kaolin. After separation of the supernatants, the adsorbates were eluted with 10 ml. of the eluent by shaking for 10 min. The proteolytic activities of the eluates were determined.

Table 2

Original solution	Increase in ml. <i>M</i> /20 KOH
Eluent:	1.25
<i>M</i> /100 acetic acid	0.20
<i>M</i> /15 KH_2PO_4	0.40
Water	0.40
<i>M</i> /15 Na_2HPO_4	0.55
1% Na_2CO_3	0.40
<i>M</i> /100 NaOH	1.00

In order to determine the minimum amount of kaolin required for adsorption of the proteolytic activity 10 ml. portions of the above-mentioned buffered dialysed culture filtrate were adsorbed with varying amounts of kaolin, eluted in each case with 10 ml. *M*/100 NaOH, and the proteolytic activity of each of the eluates estimated.

Table 3

Original solution	Increase in ml. <i>M</i> /20 KOH
g. kaolin used:	1.50
0.05	0.65
0.1	1.30
0.3	1.35
0.5	1.40
0.7	1.45

Separation of the proteolytic enzymes from the diffusing factor

A. *By acid precipitation.* To 5 ml. of a dialysed culture filtrate 0.5 ml. *M* acetate buffer pH 4.0 was added and the mixture allowed to stand overnight at room temperature. The precipitate was centrifuged off and washed with 5 ml. *M*/10 acetate buffer pH 4.0, again centrifuged and dissolved in 5 ml. *M*/10 borate buffer pH 9.2.

Table 4

	Increase in ml. <i>M</i> /20 KOH	Increase in area of spread cm. ²
Original solution	1.15	5.10
Acetic acid supernatant	0.40	4.95
Washings	0.00	0.87
Precipitate	0.70	0.65

B. *By fractional adsorption.* Both the diffusing factor and the proteolytic enzymes can be adsorbed on alumina C- γ , but the amount of adsorbent required for adsorption of the proteolytic enzymes is much smaller than for the diffusing factor. To 5 ml. of a dialysed culture filtrate 0.5 ml. *M* acetate buffer pH 4.0 was added and the mixture shaken with 0.1 ml. alumina C- γ (21 mg. $\text{Al}_2\text{O}_3/\text{ml.}$). After 10 min. the adsorbate was centrifuged off, washed with 5 ml. *M*/10 acetate buffer pH 4.0 and eluted with 5 ml. *M*/10 borate buffer pH 9.2.

Table 5

	Increase in ml. <i>M</i> /20 KOH	Increase in area of spread cm.^2
Original solution	1.85	5.69
Supernatant	0.10	4.93
Washings	0.00	0.66
Eluate	1.60	1.91

C. *By selective elution.* From adsorbates of *Cl. Welchii* culture filtrates the diffusing factor is eluted at a much lower pH than the proteolytic enzymes.

10 ml. of dialysed culture filtrate mixed with 1 ml. *M* acetate buffer pH 4.0 were shaken with 2 ml. alumina C- γ . After 10 min. the precipitate was centrifuged and eluted with 10 ml. *M*/10 Na_2HPO_4 .

Table 6

	Increase in ml. <i>M</i> /20 KOH	Increase in area of spread cm.^2
Original solution	1.35	4.26
Supernatant	0.05	1.63
Eluate	0.15	4.78

M/100 NaOH elutes from kaolin adsorbates both the diffusing factor and the proteases. 10 ml. of a dialysed culture filtrate buffered with 1 ml. *M* acetate buffer pH 4.0 were adsorbed by shaking with 0.7 g. kaolin. After 10 min. the adsorbate was centrifuged off and eluted with 10 ml. *M*/100 NaOH.

Table 7

	Increase in ml. <i>M</i> /20 KOH	Increase in area of spread cm.^2
Original solution	1.05	3.19
Supernatant	0.00	0.12
Eluate	1.10	3.62

0.4% ammonia has the same eluting properties as *M*/100 NaOH. 1% pyridine, on the other hand, elutes only the diffusing factor.

Table 8

	Increase in ml. <i>M</i> /20 KOH	Increase in area of spread cm.^2
Original solution	0.70	4.62
Supernatant	0.00	0.30
Ammonia eluate	0.50	3.92
Pyridine eluate	0.05	4.22

It was necessary to prove that pyridine has no great effect on the action of the proteolytic enzyme. For this purpose 1 ml. of dialysed culture filtrate was incubated with 1 ml. 2% pyridine for 1 hr. at 38°. The proteolytic activity of

this incubated solution was compared with that of a 1/1 dilution of the original culture filtrate. Both solutions gave approximately the same increase of carboxyl groups.

SUMMARY

Various methods are described whereby the proteases present in *Cl. Welchii* filtrates can be separated from the diffusing factor.

The author's thanks are due to Prof. A. R. Todd for his continued interest and advice. He is also grateful to Dr D. McClean of the Lister Institute (Elstree) for supplies of *Cl. Welchii* filtrates and to Imperial Chemical Industries, Ltd., for grants.

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