MANDL, I. & ZAFFUTO, S. F. (1958). *J. gen. Microbiol.* **18**, 13-15

Serological Evidence for a Specific *Clostridium histolyticum* Gelatinase

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SUMMARY: Evidence is presented that a specific gelatinolytic enzyme besides collagenase is present in purified *Clostridium histolyticum* collagenase preparations. This means that collagenase itself need not be able to digest gelatin but it does not exclude the possibility.

The purest collagenase available to date (DeBellis, Mandl, MacLennan & Howes, 1954) is free of non-specific proteolytic activity and active only against collagen and collagen degradation products (azocoll, gelatin). The question whether these activities are due to the same enzyme, i.e. whether collagenase will break down gelatin as well as collagen is still unsolved. It is possible that a series of reactions catalysed by distinct enzymes is involved but that our analytical procedures are inadequate for the detection of the primary effect of collagenase. This may involve simple breaking of hydrogen bonds as in urea treatment which results in denatured collagen susceptible to enzymes such as trypsin. However, the entire collagenase system differs from such proteinases by its specificity and complete lack of reaction with other proteins such as casein or haemoglobin as well as numerous synthetic substrates tested in this laboratory (Mandl, Zipper & Ferguson, 1957).

The presence of a specific gelatinase was first suspected when fractionation of crude collagenase (DeBellis *et al.* 1954) invariably showed the fraction with maximum collagenase activity preceding the corresponding azocoll maximum. If the collagenase were the only enzyme in this region capable of attacking azocoll, the maxima should coincide. Furthermore, the ratio of collagenase to azocoll activity is not the same in different preparations and the concentrations of certain ions (Co, Ni) needed to inhibit activity of any one preparation differ with the substrate hydrolysed (see Table 1).

Ogle & Logan (1956) have also advanced evidence for the presence of a distinct gelatinase in the collagenase system.

Gelatin or azocoll attack by the preparations investigated could not be ascribed to other known proteinases (MacLennan, Mandl & Howes, 1958; DeBellis *et al.* 1954) since the fractions were free of δ -proteinase and γ - (or ϵ -) proteinase would not be active in the absence of cysteine.

In the belief that serological tests would be more conclusive the following set of experiments was set up: varying dilutions of six sera kindly supplied by Professor Oakley of Leeds University and used for the detection of other specific enzymes (MacLennan *et al.* 1958; Oakley & Warrack, 1950) were incubated for half-hour periods with equal volumes of 0.1 % solutions of an enzyme fraction rich in collagenase and free of δ -proteinase prepared according

Table 1. *Molar concentrations of different metal ions completely inhibiting action of 0.1 mg enzyme L 139_p28 fraction III [26–30 % (NH₄)₂SO₄ conc.]*

Metal ion	Substrates		
	Collagen (10 mg./2.5 ml.)	Azocoll (50 mg./10 ml.)	Gelatin (30 mg./2 ml.)
Ca	> 0.5	10 ⁻¹	> 1
Co	0.5	10 ⁻³	> 0.5
Fe ⁺⁺	10 ⁻³	10 ⁻³	0.5
Fe ⁺⁺⁺	10 ⁻³	10 ⁻³	10 ⁻²
Mg	0.5	10 ⁻¹	> 1
Mn	0.5	10 ⁻²	> 0.5
Ni	10 ⁻³	10 ⁻³	> 0.5
Pb	10 ⁻³	10 ⁻³	10 ⁻¹
Zn	10 ⁻³	10 ⁻³	10 ⁻¹

Table 2. *Relative serum concentrations completely inhibiting action of enzyme No. L 139_p28 fraction III [26–30 % (NH₄)₂SO₄ conc.]*

Serum no.	Substrate		
	Collagen (10 mg./5 ml.)	Azocoll (10 mg./5 ml.)	Gelatin (30 mg./ml.)
RR 2001	100	100	100
RR 2005	300	200	130
RR 2008	300	400	155
RR 2035	200	200	155
RR 8003	100	300	210
RR 8006	150	200	130

to the method of DeBellis *et al.* (1954), The resulting complexes were then allowed to react with the substrates under investigation—collagen, azocoll and gelatin—and the lowest serum dilutions which completely inhibited the breakdown of these substrates compared. Azocoll and collagen tests were performed as previously described (Mandl, MacLennan & Howes, 1953; MacLennan *et al.* 1958). Gelatinase was tested by its action on 3 % gelatin in 0.1 M-phosphate buffer pH 7.1. In all tests with added metal ions (Table 1) veronal + acetate buffer pH 7.4 (Michaelis, 1931) was used instead of phosphate. After incubation at 37° for 3 hr. the tubes were placed in the refrigerator and the end-point determined visually as the lowest serum concentration preventing liquefaction. Relative results are shown in Table 2, arbitrarily assigning a value of 100 to all anti-enzyme concentrations of serum no. 2001.

Relative inhibiting concentrations for azocoll and collagen hydrolysis largely agree with those found by MacLennan *et al.* (1958) for crude collagenase, but there are some differences, i.e. relative values of serum RR 8003 for collagen and RR 2035 for azocoll, between the crude collagenase and the purified fraction used in this study. Anti-collagenase values with collagen as substrate are essentially identical with the values of Oakley & Warrack (1950). With azocoll as substrate, values differ for some sera, indicating the presence of another enzyme, besides collagenase, capable of attacking this substrate.

The newer results with gelatin as substrate show that some other enzyme besides that acting on collagen is present and active against gelatin, although

the possibility that the collagenase (β -toxin) also acts against gelatin cannot be excluded.

Table 8 shows a comparison between the end-points against azocoll and gelatin substrate of a purified δ -proteinase fraction, free from collagenase. Serum no. 2001 has again been arbitrarily assigned relative values of 100.

Table 8. *Relative serum concentrations completely inhibiting action of enzyme No. L139_p28 fraction I [0–22% (NH₄)₂SO₄ conc.]*

Serum no.	Substrate	
	Azocoll (10 mg./5 ml.)	Gelatin (30 mg./ml.)
RR2001	100	100
RR2005	50	75
RR2008	200	115
RR2035	200	170
RR8003	100	100
RR8006	100	115

The end-points agree with the δ values found with the differently prepared and far less active proteinase described by MacLennan *et al.* (1958). They are the same against both substrates within experimental error and distinct from those shown in Table 2. The gelatinase activity of this fraction can therefore be ascribed to δ -proteinase and is distinct from the new gelatinase reported here.

The findings reported increase the minimum number of proteolytic enzymes present in *Clostridium histolyticum* filtrates to seven: δ -proteinase (cysteine inhibited), γ -proteinase (cysteine activated), β -collagenase, amidase-esterase (ϵ ?), at least two peptidases (Mandl, Ferguson & Zaffuto, 1957) and the new gelatinase here reported. They also show that a specific gelatinase is associated with the purest collagenase available. Collagenase itself may or may not attack gelatin.

This work was supported in part by a grant from the United States Public Health Service, National Institute for Arthritis and Metabolic Diseases.

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(Received 20 February 1957)