The κ -Toxin (Collagenase) of *Clostridium welchii*

2. THE EFFECT OF ALKALINE pH AND HEAT

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In a previous paper (Bidwell & van Heyningen, 1948) the partial purification and characterization of the κ -toxin (collagenase) of *Clostridium welchii* was described. It was observed that dialysis of purified enzyme preparations against borate-sodium carbonate buffer at pH 10.2 resulted in an increase in the activity of the enzyme against 'azocoll' (commercial hide powder to which an azo dye has been coupled; see Oakley, Warrack & van Heyningen, 1946), and a marked decrease in the immunological test dose using azocoll as indicator. The effect of dialysis at pH 10.2 has since been repeatedly observed. In six cases there was a considerable rise in enzyme activity ('recovery' 120–210%); in four cases the activity was practically unchanged (90–110%) and in four cases there was considerable loss. In all cases we confirmed that there was a marked decrease in the immunological test dose with azocoll as indicator, but the extent of this apparent increase in combining power varied considerably ('recovery' 145–275%; mean value 188%).

It was found by Dr A. Pirie (private communication) that one of our collagenase preparations was active in disintegrating ox cornea collagen, but that a later sample of 'collagenase', which we expected from our tests on azocoll to be about as active as the first, was totally devoid of the power to digest ox cornea collagen. Reference to our records showed that this second sample had, at an earlier stage in its history, been dialyzed at pH 10.2. Several samples which had been thus treated were tested for ability to disintegrate muscle and collagen 'paper' (Oakley *et al.* 1946), and in every case it was found that although the material was still highly active in bringing about solution of azocoll, no trace of activity against muscle or collagen paper remained. Further investigations on the effect of alkaline pH on collagenase preparations are reported in the present paper.

METHODS

The activity of enzyme preparations in bringing about solution of azocoll was measured in Q units/ml. (Bidwell & van Heyningen, 1948). It must be understood that, as the work to be described will demonstrate, such enzymic activity could be due to true collagenase (i.e. unaltered collagen as specific substrate) and other enzymes which can attack azocoll but not collagen. In the sequel the term

'azocoll enzyme' will be used to denote enzymic activity measured against azocoll. Collagenase antigen was measured by its combining power in κ units/ml. by the methods of Oakley *et al.* (1946) using serum R 8537 (180 anti- κ units/ml.) as standard with (a) azocoll, (b) collagen paper, and (c) muscle as indicators; lecithinase antigen in Le units by the method of van Heyningen (1948), total nitrogen by the micro-Kjeldahl technique; pH values were measured electrometrically at 15° using a glass electrode. For the composition of the borate buffers used see Clark (1928).

EXPERIMENTAL AND RESULTS

Four separate batches of *Cl. welchii* type A crude filtrate were worked up according to the method of Bidwell & van Heyningen (1948). Table 1 shows the stages in the purification process and a comparison at each stage between the azocoll-enzyme content and the 'collagenase' antigen content as determined with the three indicators azocoll, collagen paper and muscle for a typical purification. It is probable that the first ammonium sulphate precipitation results in a change resembling toxoiding in that the enzyme activity appears to be diminished in relation to total antibody-combining power, but active enzyme is recovered preferentially to total antigen in the calcium phosphate treatment. It is clear from Table 1 that true collagenase antigen, measured using muscle or collagen paper as indicator, is retained throughout the stages of the purification.

The effect of alkaline pH on collagenase preparations

Preliminary work was done entirely on purified enzyme preparations by dialyzing against borate- Na_2CO_3 buffer at pH 10.2. It should be emphasized that in all cases the pH was restored to c. 7.4 before testing for enzyme or antigen content.

The effect on purified enzyme preparations of (a) dialyzing against borate buffers of varying pH and (b) adjusting the pH by alkali. The enzyme preparation used (B 476 G) had been purified by the method of Bidwell & van Heyningen (1948). It contained 1100 Q units of collagenase/ml., 3900 κ units of collagenase antigen/ml. (using azocoll as indicator) and 1.78 mg. non-dialyzable nitrogen/ml. For the following experiments it was diluted 1/50 in borate buffer pH 7.4. (a) Samples were dialyzed at c. 2° against seven daily changes of borate buffers as follows: Palitzsch's borate buffer pH 7.35 and 8.47; 0.05 M-sodium borate pH 9.17 and Kolthoff's borate- Na_2CO_3 buffers pH 9.59, 10.34 and 10.73, respectively, and each was then redialyzed to equilibrium against borate

Table 1. *Relation between activity of collagenase enzyme (measured in Q units/ml.) and apparent collagenase antigen content, using as indicator (a) azocoll (κ_A units/ml.), (b) collagen paper (κ_C units/ml.) and (c) muscle (κ_M units/ml.)*

Stage of purification	Q units/ ml.	κ -test doses/ml.			Relative activities			
		κ_A units/ ml.	κ_C units/ ml.	κ_M units/ ml.	κ_A units/ ml.	κ_C units/ ml.	κ_A units/ ml.	κ_M units/ ml.
		κ_A units/ ml.	κ_C units/ ml.	κ_M units/ ml.	κ_C units/ ml.	κ_M units/ ml.	Q units/ ml.	Q units/ ml.
Crude filtrate	1.67	4.2	3.9	3.1	1.1	1.4	2.5	1.8
Precipitated with $(\text{NH}_4)_2\text{SO}_4$ (60 kg./100 l.); scum dissolved in water	48	200	125	180	1.6	1.1	4.2	3.7
Adsorbed on calcium phosphate and eluted with 10% (w/v) $(\text{NH}_4)_2\text{SO}_4$ (eluate I)	19	49	31	31	1.6	1.6	2.6	1.6
Eluate I dialyzed against tap water	13.7	33	22	22	1.5	1.5	2.4	1.6
Second calcium phosphate treat- ment, giving eluate II	28.4	76	48	51	1.6	1.5	2.7	1.8
Decolorized with 1% (w/v) charcoal	32	67	40	46	1.7	1.5	2.1	1.4
Dialyzed against tap water	22	50	35	35	1.4	1.4	2.3	1.6
Solution of precipitate obtained on adding 2 vol. saturated $(\text{NH}_4)_2\text{SO}_4$ solution	466	1160	500	670	2.3	1.7	2.5	1.3
Dialyzed against borate buffer pH 7.4	355	1240	510	480	2.4	2.6	3.5	1.4

buffer pH 7.4. Volume changes were negligible (<2%). The possible specific effect of the carbonate ion introduced at the change from Palitzsch to Kolthoff buffers was considered, but, in a similar experiment the effect of dialysis against a carbonate-bicarbonate buffer of pH 9.1 (Delory & King, 1945) was shown to be the same as when 0.05M-sodium borate at the same pH was used. (b) A second set of samples of diluted enzyme preparation were adjusted with 0.1N-NaOH to pH values corresponding to those of the buffers used in the dialysis experiment. Each sample was divided into three equal parts which were then treated separately as follows: (1) Heated at 37° for 0.5 hr., cooled to 15° and pH readjusted to 7.4. (2) Left overnight at c. 2°, pH readjusted to 7.4. (3) Left 7 days at 2° and pH readjusted to 7.4.

The preparations were tested for activity against azocoll (in Q units/ml.) and for κ -antigen content using both azocoll and muscle as indicators. The results are expressed in Figs. 1, 2 and 3 as a percentage of the value for the preparation which had remained at pH 7.4 throughout the experiment. In the concentration used the enzyme appeared to be stable at pH 7.4 in all the experiments.

Where the enzyme was kept throughout in the cold, the activity against azocoll was minimal after treatment at pH 8.5-9.0, with increasing activity after treatments at pH 9-10.3 and then a very sharp drop after being held at pH 10.8 (Fig. 1). True collagenase antigen (tested against muscle, see Fig. 2) was appreciably unstable at pH 9.0 and no muscle-disintegrating activity remained after treatment at pH 10.3, since even in the total absence of antiserum the undiluted enzyme preparations were

without effect on muscle or collagen paper. The results were qualitatively the same whether the pH was changed by simple adjustment or by dialysis; they cannot, therefore, be ascribed to the removal by dialysis of some factor which dissociates from the enzyme at these alkaline pH values, but may be due to the formation from some inactive precursor of a second enzyme, which can disintegrate azocoll but not true collagen.

The effect on the apparent combining power as tested using azocoll as indicator is shown in Fig. 3.

When crude enzyme preparations were dialyzed against borate- Na_2CO_3 buffer at pH 10.2 the results were qualitatively similar to those found for purified enzyme preparations and so will not be given in detail. In all cases most of the lecithinase activity appeared to be destroyed.

The effect of heat on purified collagenase preparations. Equal volumes of a partially purified collagenase preparation diluted in borate buffer pH 7.4 were heated in a water bath at various temperatures for 10 min. and then immediately cooled. Determinations of azocoll-enzyme activity and combining power with muscle and azocoll as indicators were made. Fig. 4 shows that loss of muscle-disintegrating activity was apparent after heating to 50°, and complete after heating to 56°, but there was a slight rise in azocoll-disintegrating activity after heating to 50°, and at least 50% of the azocoll-enzyme activity remained after heating to 56°.

Fig. 1. Azocoll-disintegrating activity after alkali treatment: (a) pH changes effected by dialysis against borate buffers ($-\Delta-\Delta-$). (b) pH changes effected by addition of 0.1N-NaOH; (1) heated at 37° for 0.5 hr., $-\circ-\circ-$; (2) left overnight at c. 2°, $-x-x-x-$; (3) left 7 days at c. 2°, $-\bullet-\bullet-$. pH readjusted to 7.4 with 0.1N-HCl before testing.

Fig. 3. Apparent combining power using azocoll as indicator after alkali treatment. Symbols: as in Fig. 1.

Fig. 2. Collagenase antigen (combining power; muscle as indicator) after alkali treatment. Symbols: as in Fig. 1.

Fig. 4. The effect on the activity of purified collagenase preparations of heating for 10 min. at various temperatures. Results are shown as a percentage of values obtained for a control solution kept at room temperature during the heating experiments. Azocoll-enzyme activity, $-x-x-x-$; antibody combining power (azocoll as indicator), $-\circ-\circ-$; antibody combining power (muscle as indicator), $-\Delta-\Delta-$.

Solutions of purified collagenase preparations may undergo a similar change on long storage, since one such preparation after more than 2 years' storage at c. 2° had lost most of its muscle-disintegrating activity while retaining its ability to disintegrate azocoll. Duthie & Lorenz (1948) had previously made a similar observation; old preparations of purified collagenase which had lost their true collagenase activity still possessed high gelatinase activity.

The effect of alkali treatment on the ability of enzyme preparations to disintegrate hide powder and to split gelatin. The disintegration of commercial hide powder by an enzyme preparation which had been dialyzed at pH 10.2 was compared with that of the corresponding preparation before treatment, using the method described by Bidwell & van Heyningen (1948). The enzyme preparations were diluted to equivalent activities against azocoll. Over the range of pH tested (4.3–8.0) the amount of hide powder brought into solution by the two preparations was very nearly the same. The preparations were almost inactive at pH 4.3, but from pH 5.0 to 8.0 there was little variation in activity. Controls were run with heat-inactivated enzyme preparations as usual.

Experiments using enzyme preparations which had been dialyzed at pH 10.2 were made on gelatin, using the non-precipitable-nitrogen technique (Bidwell & van Heyningen, 1948) and also the titration of liberated $-\text{NH}_2$ and $-\text{COOH}$ groups.

Five ml. enzyme preparation (a) or (b) (see below), or the corresponding heat-inactivated enzyme for controls, were pipetted into 40 ml. gelatin (0.5% (w/v) in borate buffer pH 7.2) at 37°, incubated 1 hr. at 37°, then 5 ml. phosphotungstic acid (5% (w/v) in $\text{N-H}_2\text{SO}_4$) added, shaken, filtered after 5 min. through Whatman no. 3 papers and total-N determinations carried out on filtrates. Enzyme preparation (a) was a purified collagenase preparation which had not been treated with alkali. Enzyme preparation (b) was a similar preparation after dialysis against borate- Na_2CO_3 buffer at pH 10.2.

The activity of the two preparations against azocoll was approximately the same (5 Q units/ml.); in the experiment with gelatin preparation (a) gave 5.69 mg. N/50 ml. filtrate; (b) gave 3.69 mg. N/50 ml. filtrate.

The results show that the alkali-treated preparation was still active against gelatin, but for a given activity against azocoll it appeared to be rather less active against gelatin than the untreated preparation. Both preparations liberated equivalent amounts of $-\text{NH}_2$ and $-\text{COOH}$ groups. The results on gelatin may be complicated by the possibility that part, at least, of the gelatin-splitting activity of purified enzyme preparations may be due to yet another enzyme, neither collagenase nor the enzyme formed on alkali treatment (Bidwell & van Heyningen, 1948; and unpublished results).

Some of the earlier work on substrate specificity was repeated, using enzyme preparations before and

after treatment at pH 10.2, which were highly active against azocoll. The action on casein and urea-denatured haemoglobin was tested by a method based on that of Anson (1938), but the incubation was carried out at 37° for 0.5 hr. The untreated preparations showed only a very slight trace of activity; the alkali-treated preparations were completely inactive.

DISCUSSION

It seems reasonable to reserve the name 'collagenase' for an enzyme capable of disintegrating 'native collagen', but the decision as to what is, or is not, 'native collagen' is a matter of some difficulty (see, for example, the discussion by Kanagy, 1947). Oakley, Warrack & Warren (1948) showed that filtrates of *Cl. welchii* types B and E (Bosworth, 1943) contain a substance capable of disintegrating azocoll but not muscle or collagen paper. The foregoing experiments support their view that ability to disintegrate hide powder (or azocoll) cannot be used as a specific test for true collagenase activity (see also Todd, 1947), but providing the latter is established by other means, azocoll is still useful as a substrate in quantitative estimations. At present, in the absence of evidence to the contrary, we regard as a true collagenase an enzyme capable of disintegrating muscle and collagen paper in the way described by Oakley *et al.* (1946), as well as bringing about solution of azocoll and splitting gelatin.

The experiments cited above showed that true collagenase (active against muscle and azocoll) began to be unstable in the cold at pH 8.5–9.0 and was completely destroyed at pH 10.3. On warming to 37° destruction was greatly accelerated, so that after 30 min. at 37°, even at pH 8.5 more than 80% was destroyed. It is suggested that a second enzyme (inactive against muscle, but active against hide powder and azocoll) appears at the higher pH values, the formation being slow in the cold at pH 9 and only considerable at pH 10. At still higher pH values (beyond 10.5) this enzyme also is destroyed. Heating at 37° speeds up both the formation and destruction of this postulated second enzyme, so that minimum and maximum azocoll-enzyme activities are observed at rather lower pH values than when the treatment is carried out in the cold. Heating to rather higher temperatures (c. 45–50°) at pH 7.4 has an effect which appears to be similar to that of alkali treatment.

Since antisera, produced in response to the injection of culture filtrates of *Cl. welchii* type A, contain antibody to this second enzyme, an antigen capable of stimulating its production must be present in toxic filtrates which have not been alkali treated. This does not necessarily imply that crude culture filtrates contain the second enzyme in an active form since the zymogen may itself be able to

stimulate production of antibody capable of neutralizing the action of the enzyme.

Walbum & Reymann (1933, 1934) investigated the gelatinase of *Cl. welchii* (presumably type A) and produced evidence to show that the enzyme had two pH optima—at c. 6.0 and 8.9 respectively, with a minimum at c. pH 7.5. The enzyme was incubated with the gelatin substrate at 37° for as long as 18 hr. They defined 1 enzyme unit as that quantity of enzyme which had digested the gelatin to such an extent that the mixture had just lost the capacity to form a solid gel on cooling under standard conditions. Walbum & Reymann tended to the view that culture filtrates of *Cl. welchii* contain two gelatinases which have different pH optima. The results of the present work suggest that under the conditions they used, Walbum & Reymann may have been observing the resultant of several effects—the destruction at unfavourable pH of the 'primary' enzyme present in the original culture filtrate, the formation of the second enzyme and in addition the usual variation of enzyme activity with pH. The balance between the formation of the second enzyme at higher pH and the fact that its activity would appear from our work to be optimal at about neutral pH might very well explain Walbum & Reymann's observed second peak at pH 8.9. Their observation of a minimum at pH 6.7, i.e. lower than would be expected from the present work, may be connected with the fact that over this range they were using phosphate buffer which appears to be unfavourable to collagenase (Bidwell & van Heyningen, 1948). Jennison (1945, 1947) has shown that *Cl. histolyticum* produces a true collagenase, so that the results of Walbum & Reymann (1934), showing two pH optima for the action of *Cl. histolyticum* filtrates on gelatin, may also be due to the combination of several effects as discussed for *Cl. welchii*.

Gonzalez (1946), in his investigation of the proteo-

lytic activity of toxic filtrates of gas gangrene bacteria, conducted his enzyme reactions at 50°, which appears from the present work to be inadvisable, at least for *Cl. welchii* filtrates, since this is the temperature at which destruction of the true collagenase and formation of the second enzyme was most marked.

The immunological relation between true collagenase and the enzyme present in alkali-treated preparations is being investigated by my colleagues Dr C. L. Oakley and Miss G. H. Warrack. The results of this work will be reported separately.

SUMMARY

1. The effect of mild alkali treatment and of heat on preparations of the collagenase of *Cl. welchii* type A has been investigated. The activity of such preparations in disintegrating muscle and collagen 'paper' is readily destroyed at pH values higher than 8.5 even in the cold.
2. The effect of alkali treatment or of heating on the activity of these preparations in disintegrating hide powder and azocoll did not run parallel to the effect on the muscle-disintegrating activity.
3. It is suggested that, as a result of treatment at pH 9–10, or heating to c. 50° for 10 min. a second enzyme is formed, derived either from collagenase or some other precursor present in culture filtrates.
4. This second enzyme disintegrates hide powder and azocoll, but is not a true collagenase, since it is without action on collagen paper and muscle. It is destroyed at pH values beyond 10.5 or by heating to 60° for 10 min.

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REFERENCES

- Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
 Bosworth, T. J. (1943). *J. comp. Path.* **53**, 245.
 Bidwell, E. & van Heyningen, W. E. (1948). *Biochem. J.* **42**, 140.
 Clark, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed. London: Baillière, Tindall and Cox.
 Delory, G. E. & King, E. J. (1945). *Biochem. J.* **39**, 245.
 Duthie, E. S. & Lorenz, L. (1948). *Biochem. J.* (in the Press).
 Gonzalez, A. A. (1946). *Rev. Fac. Cienc. quim. La Plata*, **19**, 93.
 Jennison, M. W. (1945). *J. Bact.* **50**, 369.
 Jennison, M. W. (1947). *J. Bact.* **54**, 55.
 Kanagy, J. R. (1947). *Circular C 458 National Bureau of Standards*. Washington: U.S. Government Printing Office.
 Oakley, C. L., Warrack, G. H. & van Heyningen, W. E. (1946). *J. Path. Bact.* **58**, 229.
 Oakley, C. L., Warrack, G. H. & Warren, M. E. (1949). *J. Path. Bact.* **61** (in the Press).
 Todd, E. W. (1947). *J. exp. Med.* **85**, 591.
 van Heyningen, W. E. (1948). *Biochem. J.* **42**, 127.
 Walbum, L. E. & Reymann, G. C. (1933). *J. Path. Bact.* **36**, 469.
 Walbum, L. E. & Reymann, G. C. (1934). *J. Path. Bact.* **39**, 669.