
**New Proteolytic Enzymes from Clostridium histolyticum Filtrates**

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**SUMMARY:** Oakley & Warrack (1950) established the presence of two serologically distinct proteolytic enzymes in *Clostridium histolyticum* filtrates, a β-toxin digesting native collagen and a cysteine-activated γ-toxin attacking azocoll and other protein substrates.

In the present paper we give evidence of another proteolytic enzyme, not activated by cysteine and serologically different from those previously described. This we designate as δ-toxin. We also indicate the possibility of the presence of a fourth enzyme more stable than the others and active against various synthetic substrates in conjunction with cysteine. This enzyme could not be inhibited by antisera. Furthermore peptidases have been detected and will be described in detail elsewhere.

Some of the differences between these enzyme systems are discussed.

Several papers have been published on the various toxins and enzymes present in culture filtrates of *Clostridium histolyticum*, the most important studies being those of Oakley & Warrack (1950) who were able by serological methods to define three factors: the α-toxin, which is the main lethal toxin, the β-toxin, a collagenase, and the γ-toxin, which is a cysteine-activated proteinase. Bowen (1952) and Howard (1953) published evidence for a distinct haemolysin component antigenically related to *C. welchii* θ-toxin and to tetanolysin. There has also been considerable interest in the biochemical nature of the proteolytic enzymes. Since the demonstration by Jennison (1947) that culture filtrates of *C. histolyticum* were collagenolytic some degree of concentration of the collagenase has been obtained by various methods (Tytell & Hewson, 1950; Mandl, MacLennan & Howes, 1958; DeBellis, Mandl, MacLennan & Howes, 1954) but the exact nature of this enzyme remains uncertain. On the other hand, very considerable purification of the cysteine-activated proteinase (the γ-toxin) has been achieved (Kocholaty & Krejci, 1948; Lepow, Katz, Pensky & Pillemer, 1952; Ogle & Tytell, 1953). All these studies confirm that this enzyme is cysteine-activated and readily inhibited by iodoacetamide and similar compounds. It was therefore with considerable surprise that in the course of our investigations of clostridial proteinases (MacLennan, Mandl & Howes, 1958; Mandl, MacLennan & Howes, 1958) we separated from *C. histolyticum* filtrates a proteinase which was not activated by –SH compounds. In a previous publication (Mandl *et al.* 1953), we briefly mentioned that this new enzyme was distinct from either the β- or γ-toxins of Oakley (Oakley & Warrack, 1950) and suggested for it the name ‘δ-toxin’. The present paper is concerned with a more detailed account of these and subsequent studies.

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RESULTS

Professor C. L. Oakley very kindly put at our disposal six sera (2001, 2005, 2008, 2035, 8003, 8006) which had been calibrated by him (Oakley & Warrack, 1950). These sera in various dilutions were incubated for 30 min. at 37° with solutions of different enzyme preparations. The mixtures were then added to 10 mg. amounts of azocoll (Oakley, Warrack & van Heyningen, 1946) and the volumes made up to 10 ml. with phosphate saline buffer (pH 7.4) by an adaptation of the procedure of Oakley et al. (1946). The colour released was read in a Klett colorimeter after 24 hr. of incubation at 37° and the units required for complete inhibition calculated by comparison with one serum (2001), to which a value of 100 enzyme units had arbitrarily been assigned. Table 1 shows the values so obtained against two different proteinase preparations (Mandl et al. 1953) as compared with the anti-β and anti-γ values obtained by Oakley & Warrack (1950) with these same sera. Almost the same relative figures were obtained when azocoll tests were run with 50 mg. amounts of substrate and correspondingly higher enzyme concentrations on a Fisher Gyrosolver shaking machine for 15 min. Professor Oakley has since very kindly checked our results and essentially confirmed them. A more detailed report of his investigations is given in the following paper (Oakley & Warrack, 1958).

Table 1. Values of antisera against proteinase preparations (anti-δ units) compared with Oakley's anti-β and anti-γ units

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Anti-δ units against proteinase no. 199</th>
<th>Anti-δ units against proteinase no. 245</th>
<th>Anti-β units Oakley &amp; Warrack (1950)</th>
<th>Anti-γ units Oakley &amp; Warrack (1950)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR2001</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>RR2005</td>
<td>75</td>
<td>50</td>
<td>350</td>
<td>100</td>
</tr>
<tr>
<td>RR2008</td>
<td>170</td>
<td>150</td>
<td>370</td>
<td>360</td>
</tr>
<tr>
<td>RR2035</td>
<td>200</td>
<td>175</td>
<td>230</td>
<td>150</td>
</tr>
<tr>
<td>RR8003</td>
<td>120</td>
<td>125</td>
<td>260</td>
<td>550</td>
</tr>
<tr>
<td>RR8006</td>
<td>120</td>
<td>100</td>
<td>180</td>
<td>410</td>
</tr>
</tbody>
</table>

Table 1 clearly shows that the main component of our proteinase preparations [P199, P245] (Mandl et al. 1953) is not identical with either the β- or γ-toxin of Oakley & Warrack (1950). We proposed the designation of δ-toxin for this enzyme (Mandl. et al. 1958).

Similar tests were set up to determine the immunological identity of the collagenase in our collagenase preparations [K240, K265, K210] (MacLennan et al. 1958; Mandl et al. 1958). For these studies, in addition to azocoll, purified collagen (Einbinder & Schubert, 1951) was used as a specific substrate, complete inhibition of digestion being taken as the end-point. As will be seen from Table 2, values not too different from Oakley's anti-β figures (Oakley & Warrack, 1950) were obtained.

With azocoll as a substrate on the other hand (last column of Table 2), only four of the six sera show end-points within the range of experimental error.
for β values. Sera nos. 2005 and 2035 approached γ end-points far more closely. Sera nos. 2001 and 2008 may of course be β or γ, though sera nos. 8008 and 8006 could never be γ (Table 1). It is not surprising that the antibody present in lesser amounts (β in no. 8003 or no. 8006, γ in no. 2005 or no. 2035) should determine the end-point, if a mixture of these enzymes is assumed. There are no δ end-points with these preparations and the relative values are the same for a crude collagenase with 35–40 % proteinase (δ) and purified material completely free of activity against casein, though not against benzoyl arginine amide. (See below and DeBellis et al. 1954).

Table 2. Values of antisera against collagenase preparations compared to anti-β units

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>Collagen substrate against collagenase no. 240</th>
<th>Anti-β Oakley &amp; Warrack (1950)</th>
<th>Azocoll substrate against collagenase no. 265</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR 2001</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>RR 2005</td>
<td>400</td>
<td>350</td>
<td>125</td>
</tr>
<tr>
<td>RR 2008</td>
<td>350</td>
<td>370</td>
<td>400</td>
</tr>
<tr>
<td>RR 2035</td>
<td>300</td>
<td>230</td>
<td>75</td>
</tr>
<tr>
<td>RR 8003</td>
<td>250</td>
<td>260</td>
<td>200</td>
</tr>
<tr>
<td>RR 8006</td>
<td>220</td>
<td>180</td>
<td>150</td>
</tr>
</tbody>
</table>

Chemical tests confirmed that more than one enzyme distinct from collagenase was present in our proteinase preparations. Three typical experiments may be described.

(a) When the enzyme preparation was heated to 56° in a water-bath for 30 min., activity against azocoll was decreased to about one-third of its original value. Neither of these activities was affected by adding cysteine or iodoacetate in final concentrations of less than 0·01 M, while larger amounts of cysteine inhibited both the heated and unheated sample. Activity against collagen was lost almost completely by this heat treatment. On the other hand, activity against the synthetic substrate benzoyl arginine amide (B.A.A.) and arginine methyl ester (A.E.) was retained without any loss. These substrates were attacked only in the presence of cysteine, and optimum conditions were the same before and after heating. Table 3 gives the values obtained in two typical experiments.

Table 3. Loss in enzymic activity after heating proteinase and collagenase preparations 30 min. at 56°

<table>
<thead>
<tr>
<th>Enzyme preparations</th>
<th>Azocoll</th>
<th>Collagen</th>
<th>Benzoyl arginine amide</th>
<th>Arginine ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>P 199</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P 199 heated</td>
<td>38</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>K 210</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K 210 heated</td>
<td>12</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Our methods for carrying out these tests were described in greater detail elsewhere (Mandl et al. 1958).
The amount of collagen digested was measured by a colorimetric ninhydrin reaction with the supernatant fluid (Harding & MacLean, 1915; Harding & Warneford, 1916). Benzoyl arginine amide was split by the enzyme into benzoyl arginine and ammonia, the ammonia being then trapped in boric acid by Conway diffusion (Conway & Byrne, 1933) and estimated with Nessler's reagent (Mandl & MacLaren, 1949). In the case of arginine methyl ester the amount of unchanged ester substrate was determined by Hestrin's alkaline hydroxylamine method (Hestrin, 1949).

The difference in stability of the various factors indicates the presence of at least two enzymes. It seemed possible that this more stable enzyme, active against the synthetic substrates in the presence of cysteine, might be the γ-toxin (Oakley & Warrack, 1950). This hypothesis was tested by repeating the serological experiments outlined on page 2 of this paper, using heated enzyme against the six sera put at our disposal by Professor Oakley, with and without added cysteine. In typical experiments half as much serum was required for the neutralization of the azocoll activity of \( \text{P 199} \) after heating than before heating but comparison of the end-points obtained gave exactly the same relative values as those shown for δ-enzyme in Table 1. Unlike Oakley & Warrack, (1958), we did not find that the presence or absence of cysteine made any difference in the relative units required for complete inhibition.

(b) Differential inactivation very similar to that obtained on heating was also observed after lowering the pH value of enzyme solutions to pH 4.5 (in \( \text{KH}_2\text{PO}_4 \)). leaving it at that pH value for 1 hr. or overnight, then adding \( \text{Na}_2\text{HPO}_4 \) to bring the pH value back to 7.4 and retesting. Again azocoll activity decreased to one-third or less, while the activity against the synthetic substrates was retained in full. This experiment was also carried out with a crude \( \text{Clostridium histolyticum} \) Seitz filtrate, the pH value being decreased by adding HCl. This preparation lost all its collagenase activity, and almost all its azocoll activity, while benzoyl arginine amide was hydrolysed as before. The result appears of interest also in view of the fact that Lepow et al. (1952) made use of low pH values in the precipitation of their proteinase (presumably γ). This seems further proof that the main constituent of our enzyme cannot be γ-toxin but must be some other more labile enzyme (δ). Whether the remaining activity against benzoyl arginine amide is due to γ seems doubtful (DeBellis et al. 1954). However, the small amount of azocoll activity remaining was not increased by adding cysteine, and this fact as well as the end-points obtained in serological tests were not in favour of such an assumption. Hydrolysis of protein substrates other than azocoll, e.g. casein, was retarded in the same way as with azocoll and were also unaffected by cysteine. Table 4 shows some comparative results. Recently we have obtained evidence that fractionation of crude collagenase preparations gave fractions of maximum activity against benzoyl arginine amide, that did not attack casein (or haimoglobin). Such fractions sometimes attacked casein in the presence of added cysteine, probably due to the presence of small amounts of γ-enzyme. Similarly, purified β-enzyme (collagenase) had no effect on these two substrates (DeBellis et al. 1954).
Table 4. Loss in enzymic activity of a proteinase preparation after heating or acidification, tested with and without added cysteine

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Casein</th>
<th>Azocoll</th>
<th>Benzyol arginine amide</th>
<th>Arginine ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme 199</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ Cysteine</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Heated 30 min. 56°</td>
<td>62</td>
<td>34</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Heated + cysteine</td>
<td>58</td>
<td>34</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>pH 4.5 24 hr.</td>
<td>48</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 4.5 + cysteine</td>
<td>47</td>
<td>16</td>
<td>100</td>
<td>90</td>
</tr>
</tbody>
</table>

Casein hydrolysis was tested by a modification of Grob's method (1946) of determining the amount of undigested casein by the turbidity resulting when a sample was added to a mixture of 20% sulphosalicylic acid:25% HCl (2:1). Essentially the same serological tests as those already described for the heated enzyme were performed with proteinase preparation which had been partly inactivated by decreasing the pH value. The results again seemed to indicate the δ end-points.

(c) When a 1% solution of the same proteinase preparation percolated through an ion exchange resin (Rohm and Haas XE 64), the resulting percolate did not show any activity against azocoll but against benzoyl arginine amide and arginine ester activity was as high as that of the original solution. Addition of cysteine, Fe++, Fe+++cysteine or Ca+++ to the percolate had no effect on its ability to digest azocoll nor could this activity be recovered by mixing the percolate and an eluate obtained by passing 10% NaCl through the column. Analysis for protein showed no substantial loss following passage through the column, so that partial inactivation rather than separation seemed to have taken place.

Collagenase solutions subjected to the same treatment retained some activity (one-third to two-thirds) against azocoll, but lost nearly all their collagenolytic powers.

To summarize conclusions drawn from these three experiments, it seems obvious that the main constituent of our preparations is less stable than that described by other workers (Lepow et al. 1952; Ogle, 1952). Ogle (1952) reported his cysteine-activated proteinase to be stable at 50° for 30 min. and as mentioned above, Lepow et al. (1952) precipitated their enzyme at pH 4.5. γ-Toxin differs from ours (δ) both serologically and in its susceptibility to activators and inhibitors. While hydrolysis of azocoll or casein by our proteinase is inhibited or unaffected by cysteine or Fe++, Ca+++ has a strong activating effect (Mandl et al. 1953).

So far as we know, the identity of the cysteine activated enzyme (Kocholaty & Krejci, 1948; Lepow et al. 1952; Ogle & Tytell, 1953) with each other and Oakley's γ-toxin has not been tested by serological means.

Our enzyme preparations show cysteine-activation only towards synthetic substrates and the factor responsible for this activity is relatively stable and
distinct from the δ-enzyme. In fractionation experiments it accompanies
the collagenase (β) rather than δ-proteinase (DeBellis et al. 1954). Loss in
activity cannot be accounted for by collagenase instability alone since
collagenase-free proteinase behaves in the same way. In at least some cases all
azocoll activity is lost without affecting the activity against benzoyl arginine
amide and arginine ester; since γ-enzyme attacks azocoll, the stable factor is
probably not γ. To our knowledge, all known proteinases are active against
azocoll.

Many attempts were made to show the presence of trace amounts of γ-toxin.
The neutralization points of the six sera against proteinase plus cysteine and
against proteinase plus iodoacetic acid were investigated in the hope of getting
some indications on this point. We have to admit, however, that some of these
titrations were very erratic and had to be discarded, and even at best end-
points were not as consistent as those obtained with the enzyme alone. So far
as conclusions can be drawn, there was no marked difference between the rela-
tive amounts required in each series, and neutralization points were approxi-
mately the same and essentially δ end-points. We understand that Professor
Oakley in his more extensive investigations did observe differences pointing
to the presence of traces of γ and that he will report on his findings in the
following paper (Oakley & Warrack, 1958). We can detect a cysteine-activated
casein-splitting enzyme, possibly γ, in fractions free of δ-proteinase, usually
precipitated with collagenase in ammonium sulphate fractionations (DeBellis
et al. 1954).

The question of identity of the benzoyl arginine amide hydrolysing enzyme
with Oakley’s γ-toxin would have been amenable to serological solution if the
end-points for the inhibition of its action against synthetic substrates by
various sera could have been compared. Experiments were set up to this end,
but it was soon found that even very large excess of antiserum had no effect
on these reactions. Other cases are known in which enzymes (e.g. urease)
cannot be inhibited by their antisera, even where definite enzyme-antienzyme
complexes are formed; for a review see Cinader (1953). Bidwell & van
Heyningen (1948) observed a similar case with C. welchii κ-toxin; the action
of this enzyme against gelatin was not affected by sera inhibiting collagen and
azocoll activity. Our gelatinase, however, can be inhibited by sera (Mandl
& Zaffuto, 1958). Bidwell & van Heyningen, after considering possible reasons,
found the most likely explanation in the fact that the comparatively small
molecules of gelatin were not prevented from reaching the catalytic centre
of the enzyme, whereas the gross particles of collagen or azocoll were held back.
Such a conclusion could account for the failure of our sera to inhibit the hydro-
lysis of the much smaller benzoyl arginine amide and arginine ester molecules.
It also agrees with the statements of Cinader (1953) and Marrack (1950) that
the probability of inhibition is greater the larger the substrate. The small
size of the synthetic substrate molecule would then be the cause of the lack
of inhibition. This explanation obviously does not answer the question whether
a new enzyme is involved.

However, if all the findings are considered together, it seems likely that a
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distinct fourth enzyme (ε?) is responsible for the amidase-esterase activity, irrespective of whether γ is present in traces or not. One or more further components have recently been detected, both chemically (DeBellis et al. 1954) and serologically. They attack a variety of synthetic peptide substrates and are described in detail elsewhere (Mandl et al. 1957).

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