

ISOLATION AND CHARACTERIZATION OF PROTEINASE AND COLLAGENASE FROM *CL. HISTOLYTICUM*^{1, 2}

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INTRODUCTION

Reports on the nature of proteolytic enzymes obtained from *Cl. histolyticum* have been contradictory. Maschmann (1) described two extracellular and one intracellular enzymes besides peptidases. His extracellular proteinases are inhibited by cysteine, Fe⁺⁺, Cu, Hg and cysteine plus Fe⁺⁺. Weil and Kocholaty (2), on the other hand, found a proteinase activated by heavy metals and SH-compounds. Van Heyningen (3) tried to reconcile both findings by postulating two proteinases, one activated by cysteine, the other inhibited, so that the addition of cysteine to a system containing both enzymes would be without obvious effect. More recently, Kocholaty and Krejci (4) found that by salt precipitation they obtained one single proteinase only, activated by Fe⁺⁺ and cysteine. Collagenase activity of *Cl. histolyticum* has been reported by Jennison (5), Evans (6), Oakley and Warrack (7), and Neumann and Tytell (8). Tytell and Hewson (9) prepared both a collagen-digesting enzyme inhibited by Fe⁺⁺ and cysteine and a proteinase resembling that of Kocholaty and Krejci (4) and Van Heyningen (3). A similar proteinase has now been isolated by Lepow, Katz, Pensky, and Pillemer (10) except that their purified enzyme is optimally activated by cysteine alone, Fe⁺⁺ being without effect.

Our own studies of *Cl. histolyticum* enzymes have led us to the conclusion that all strains elaborate a collagenase and at least one other proteolytic enzyme. The amounts vary with the strains and with the medium used. Elsewhere (11) the screening of organisms was described for the selec-

tion of strains capable of producing large amounts of potent enzymes that digested native and denatured collagen. By differential precipitation techniques we have now been able to isolate some of these enzymes from media growing certain of these strains.

Precipitation of proteinase

Optimum growth conditions for proteinase production are described in a previous paper (11). After 17 hours incubation the fluid medium containing the enzyme is freed of bacteria by passage through a large Seitz filter. It is then subjected to fractional precipitation under controlled conditions of temperature, pH and ionic strength. Methanol precipitation gives a more active and purer proteinase than precipitation with acetone, ethanol, propanol and butanol. Relatively high concentrations of 66 to 72 per cent methanol, pH of 6.1 to 6.2 and high ionic strength of 0.27 were found most satisfactory (see Tables I and II). At high ionic strength only traces of collagenase are co-precipitated.

The cooled filtrate adjusted to pH 6.1 is precipitated at -5° C. with 2 to 2½ vol. methanol precooled to -15 to -20° C. The precipitate is allowed to settle overnight at a temperature of -15° C., then spun off in a refrigerated centrifuge. It is resuspended in a small amount of water, dialyzed, clarified by filtration or centrifugation and lyophilized to a white powder.

The small amount of collagenase co-precipitated under these conditions comes down with the first 1½ vol. methanol. When this fraction was centrifuged off and a further volume of methanol added to the supernatant, the second precipitate so obtained was a proteinase of high activity and completely free of collagenase (Fraction II). Although these enzyme preparations appear electrophoretically homogenous (Figure 1), they contain relatively large amounts of a saccharide im-

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TABLE I*
Precipitation of *Cl. histolyticum* proteinase
under varying conditions

Vol. CH ₃ OH	pH	Azocoll test	Casein test (15)
$\frac{1}{2}$	7.5		no ppt
$\frac{3}{4}$	7.5		no ppt
1	7.5	52	85
$1\frac{1}{2}$	7.5	70	90
2†	7.5	170	197
$1\frac{1}{2}$	5.5	19	84
$1\frac{1}{2}$ †	6.0	60	180
$1\frac{1}{2}$	6.6	43	175
$1\frac{1}{2}$	7.0	38	139
$1\frac{1}{2}$	7.5	35	90
$1\frac{1}{2}$	8.4	25	90
2†	6.1	520	268
2	6.3	199	158
2	6.55	186	155
2	6.65	143	142
2	6.85	157	152

* Azocoll and Casein digestion values are given as comparative colorimetric readings per unit volume.

† Optimal conditions.

purity which was found to be Blood Group A substance, co-precipitated from the peptone in the medium. This anti-hemagglutination factor, which is present in relatively large amounts in most mammalian tissue digests, is not precipitated to the same extent by $(\text{NH}_4)_2\text{SO}_4$. The amount of contaminant was, therefore, substantially reduced by preliminary precipitation of both enzymes with $(\text{NH}_4)_2\text{SO}_4$. Forty per cent $(\text{NH}_4)_2\text{SO}_4$ precipitated collagenase containing 20 to 40 per cent proteinase activity (35 per cent by weight). This proteinase could be separated by methanol precipitation of the dialysed collagenase preparation at

pH 7.5 in the presence of enough MgSO_4 to raise the ionic strength to 0.27. The resulting enzyme preparation was free of collagenase and very active. Experiments on the further purification of this proteinase both by chemical and physico-chemical means are in progress.

Precipitation of collagenase

Precipitation of the clear Seitz filtrate with solid $(\text{NH}_4)_2\text{SO}_4$ gives a very active enzyme preparation. Most of the activity is in a first fraction obtained by adding 400 Gm. solid $(\text{NH}_4)_2\text{SO}_4$ to each liter of filtrate, allowing it to stand at 4° C. overnight, filtering, resuspending the precipitate in a little water, dialysing, filtering off any insoluble residue and freeze drying. Further purification may be obtained by calcium phosphate adsorption and elution with 10 per cent $(\text{NH}_4)_2\text{SO}_4$ or by a second $(\text{NH}_4)_2\text{SO}_4$ precipitation at more alkaline pH, but in our hands these steps entail considerable losses. The dried enzyme is not free of proteinases.

An alternative method is CH_3OH precipitation of the dialyzed Seitz filtrate essentially as described by Tytell and Hewson (9).

Assay methods employed

Azocoll: Various modifications of the method of Oakley, Warrack, and Van Heyningen (12) estimating the release of color from Azocoll (hide powder coupled to an azo dye) have been used to determine total proteolytic activity at consecutive steps of purification.

For quantitative determinations 50 mg. Azocoll are suspended in 9.9 ml. Phosphate Buffer pH 7.4 containing 4 parts M/15 Na_2HPO_4 , 1 part M/15 KH_2PO_4 and 5 parts 0.9 per cent NaCl. One-tenth ml. enzyme solution is added and the flasks shaken for exactly 15 minutes on a Fisher Gyrosolver shaking machine placed in an incu-

TABLE II*
Effect of ionic strength and ions present on enzyme activity of
precipitate (pH 6.1, 2 vol. CH_3OH added)

Ions present	MgSO ₄ added (mg.)	Ionic strength	Azocoll test	Casein test (15)	B.A.A. test (17)
Fe ⁺⁺	0	0.1	166	238	77
Fe ⁺⁺	32	0.112	106	197	92
Fe ⁺⁺	160	0.16	169	252	206
Fe ⁺⁺	320	0.22	206	286	280
Fe ⁺⁺	0	0.1	709	208	210
Mn ⁺⁺	0	0.1	690	185	240
Mg ⁺⁺	0	0.1	520	171	160
Co ⁺⁺	0	0.1	510	149	240

* Azocoll, Casein and Benzoyl Arginine Amide digestion values are given as comparative colorimetric readings per unit volume.

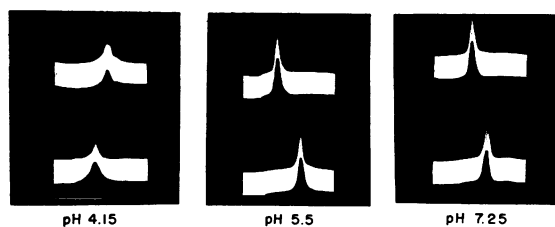


FIG. 1. ELECTROPHORETIC PATTERNS OF *CL. HISTOLYTICUM* PROTEINASE

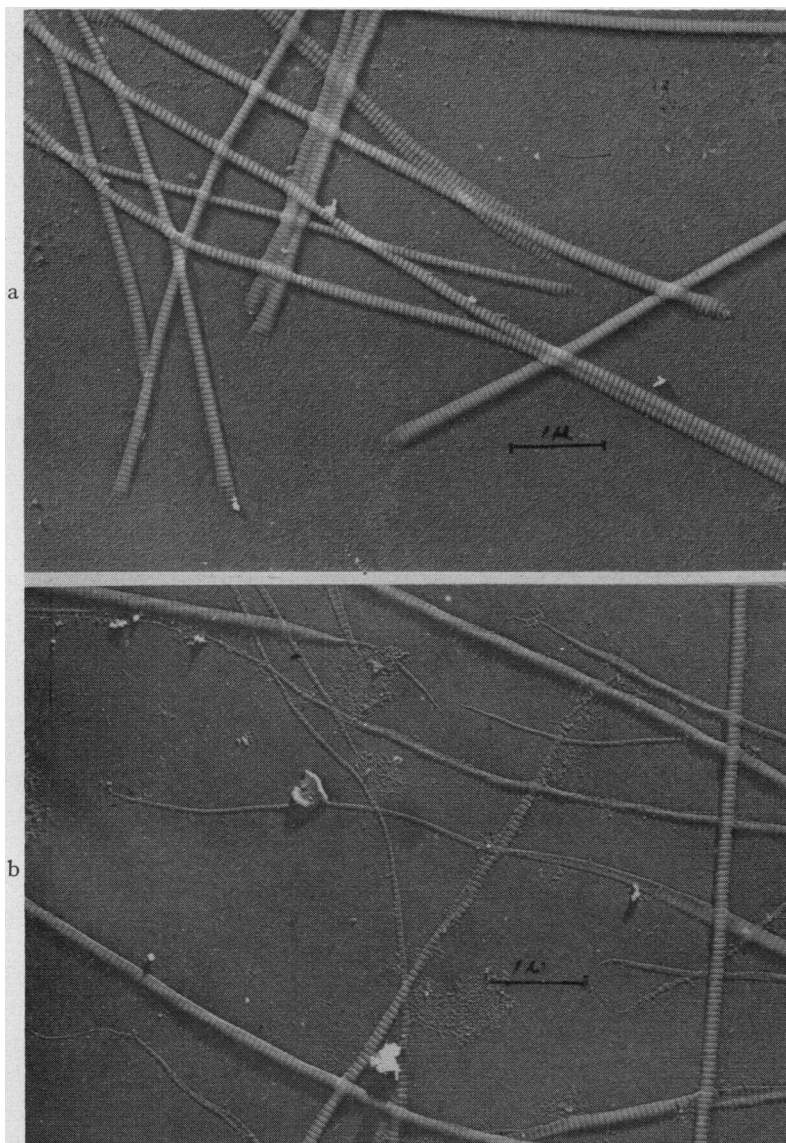


FIG. 2. ELECTRON MICROGRAPHS SHOWING EFFECT OF COLLAGENASE ON COLLAGEN FIBRILS. a. UNTREATED FIBRILS. b. COLLAGENASE TREATED FIBRILS (Preparation by Dr. Jerome Gross, Mass. Inst. of Technology)

bator at 37° C. The Azocoll powder is then filtered off through a rapid filter paper directly into Klett tubes and the color released read in a colorimeter with a green filter No. 54. The Klett reading minus the blank is calculated for 1 mg. dry enzyme. This value indicates Q units for this enzyme preparation. When the effect of added activators, inhibitors, metal salts, etc. is tested the amount of buffer is decreased to give a total volume of 10 ml.

Native collagen: Unlike Azocoll or hide powder, which are attacked by collagenase and protein-

ase(s), "undenatured" collagen is a specific substrate for collagenase. Collagenase activity was therefore determined by the action of the enzyme on Achilles tendon or collagen purified by extraction with Na_2HPO_4 and KCl to remove soluble proteins and mucopolysaccharides (13). The degree of hydrolysis was established by a modification of the ninhydrin color reaction (14) for free amino groups and Kjeldahl nitrogen tests or by serial dilution experiments. The extent of the

breakdown of collagen by collagenase was estimated by occasional Van Slyke ninhydrin CO_2 tests (15) to distinguish between peptides and free amino acids. These tests showed that at least some amino acids were released from collagen, although enzymatic digestion was not as complete as acid hydrolysis. Paper chromatograms of enzymatic and acid collagen hydrolysates confirmed these findings. Since all our collagenase preparations contain some proteinase this enzyme may be responsible for the liberation of free amino acids after breakdown to the peptide level by the collagenase. We are still engaged in the elucidation of this point.

Dr. Jerome Gross of Massachusetts General Hospital has studied the changes in the fine structure of undenatured collagen fibrils as influenced by collagenase and proteinase under the electron microscope. The proteinase either with or without cysteine activation did not attack collagen fibrils. On the other hand, the attack brought about by collagenase was very striking. The fibrils became distinctly tapered and lost their cross striations. Trypsin fragments the fibril transversely

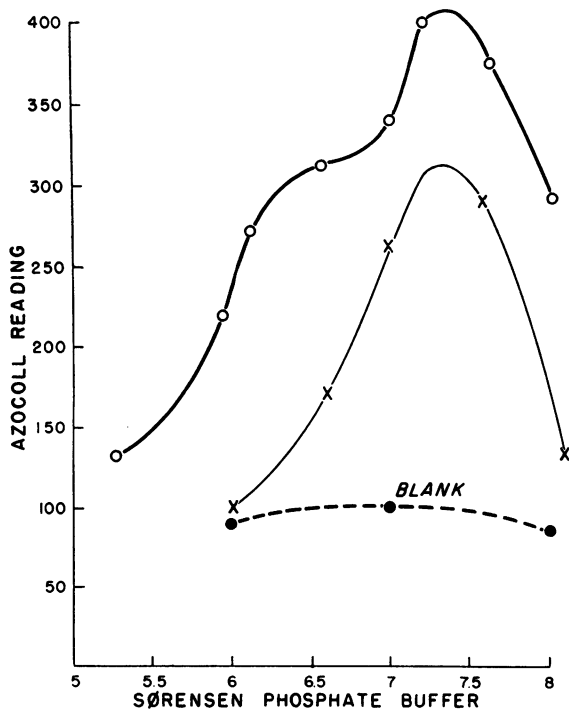


FIG. 3. ACTIVITY OF COLLAGENASE (O) AND PROTEINASE (X) IN SØRENSEN PHOSPHATE BUFFER

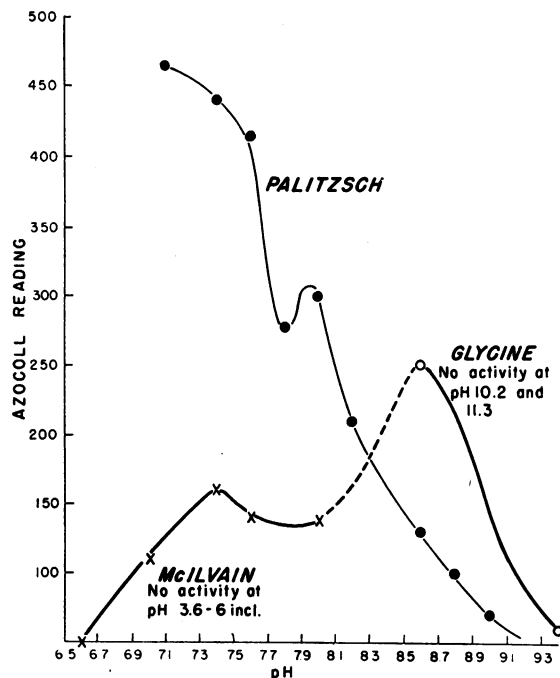


FIG. 4. ACTIVITY OF PROTEINASE IN PALITZSCH, MCILVAIN, AND GLYCINE BUFFER

but collagenase reduced the entire diameter as if it were washed away (Figure 2).

Other tissue components: None of our preparations has had any activity against elastin or keratin.

Other substrates: Enzymatic activity of our preparations was also measured against various other substrates such as casein, egg albumen, bovine serum albumen, hemoglobin, etc. by adaptation of Kunitz' method (16) of determining the tyrosine and tryptophane content of the supernatant obtained on trichloroacetic acid precipitation of the digest. For casein hydrolysis the decrease in turbidity resulting on addition of aliquots to sulfosalicylic acid at given intervals according to Grob (17) was also followed. In addition, synthetic substrates such as benzoyl arginine amide, benzoyl arginine isopropyl ester and arginine methyl ester were employed. No activity was found against tyrosine ester or glycine ester. Activity against benzoyl arginine amide was measured by Conway microdiffusion of the NH_3 released on splitting of the amide bond followed by nesslerization or titration by the method described for ficin (18). Hestrin's method (19) of reac-

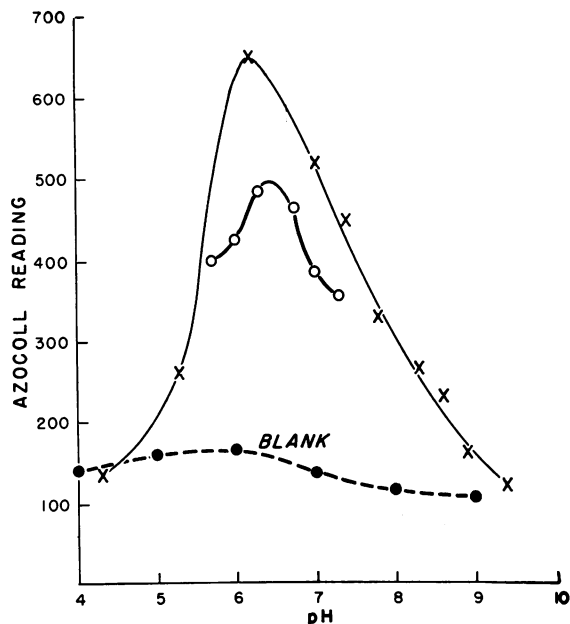


FIG. 5. ACTIVITY OF PROTEINASE IN UNBUFFERED SOLUTION AT DIFFERENT pH ESTABLISHED BY ADDING NaOH OR HCl

tion with alkaline hydroxylamine was used to follow the decrease in unsplit ester present after enzymatic interaction.

Chemical properties of proteinase and collagenase

Both enzyme preparations are typical proteins, and give positive reactions with all the usual protein reagents. There is no evidence of free SH groups as judged by the nitroprusside reaction, although S is found on ashing. Their tyrosine and tryptophane content as determined by spectrophotometric analysis is low. These findings agree with those of Kocholaty and Krejci (4). The amino acid composition has been estimated by qualitative paper chromatography. It resembles that of collagen in both enzyme preparations. Quantitative analyses have been postponed until the purer enzymes are available.

In phosphate buffers both enzyme preparations showed maximum activity at pH 7.4 (Figures 3, 4) while with other buffers different maxima were obtained (Figure 4). Activities at a given pH also varied with the buffer employed. These differences are probably due to inhibition by some of the ions present—*e.g.*, borate and citrate (Figure 4).

The collagenase-free proteinase was more thoroughly investigated first. Hence, the rest of the discussion about the factors that influence activity will be largely about proteinase.

In McIlvain phosphate-citrate buffer no proteinase activity was discerned below pH 6.6 (Figure 4); in Sørensen phosphate buffer, while the maximum activity occurred at the same pH, the enzyme was active over a much wider range (Figure 3). An apparent second maximum in glycine buffer at pH 8.6 was probably caused by the difference in buffer composition (Figure 4). In Palitzsch borate buffer activity decreased with rising pH, with a possible slight maximum at pH 8 (Figure 4). When no buffer was used and pH adjusted with HCl and NaOH only, the maximum appeared at pH 6.4 (Figure 5). It is of interest in this connection that in 1934, Walbum and Reymann (20) found two maxima, one between pH 6 to 7 and one between pH 8 to 9, for optimum gelatinase activity of crude *Cl. histolyticum* filtrates. Since they too had to change the buffer systems from citrate (pH 4 to 5) to phosphate (pH 6 to 7) to borate (pH 8 to 10) the different effects of these anions may account for their results.

Stability at different pH values also differs with the buffer system employed. At low pH, stability is greater in acetate than in McIlvain buffer (Figure 6). For at least one month stability of dilute solutions is fair over a pH range from about 5.6

STABILITY OF J PROTEINASE AT DIFFERENT pH

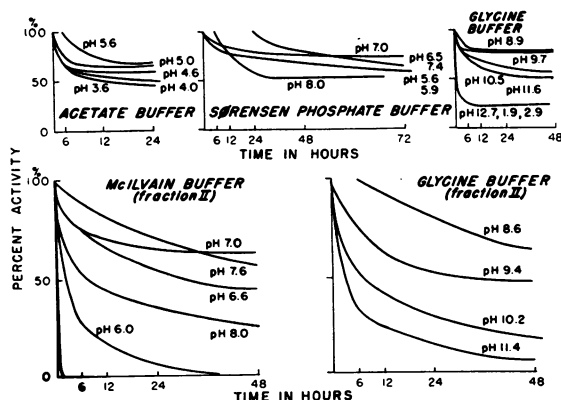


FIG. 6. STABILITY OF PROTEINASE AT DIFFERENT pH'S AND IN DIFFERENT BUFFERS

Fraction II is a purer preparation.

to high alkalinity, although stability is less for the more active highly purified preparations (Fraction II, Figure 6). In the dry state the lyophilized enzymes retain their activity indefinitely.

Table III shows the effect of various cations on proteinase activity. Hg^{++} is seen to inhibit the enzyme completely, Ag considerably, most metal ions reduce proteinase activity to some extent, but Ca^{++} increases it almost twofold; Mg, Co, and Mn activate slightly. Again, results differ somewhat with the system used, substrate and buffer (Table III).

The effects of oxidizing and reducing agents have been studied, but we were unable to confirm the cysteine activation or iodoacetamide inhibition claimed for *Cl. histolyticum* proteinase (2, 4, 7, 9, 10), except for the splitting of the synthetic substrates. At various concentrations and under various conditions of incubation, essentially unchanged activity toward Azocoll and casein was obtained in the presence or absence of cysteine or iodoacetamide. Very high concentrations of cysteine (M/10) showed definite inhibition of all enzyme preparations. Cysteine plus a heavy metal did not change the effect of the metal alone, except in the case of Co where the slight increase of activity with Co alone was no longer observed. The lack of cysteine activation led to the belief that we may be dealing with an enzyme distinct from the cysteine activated proteinase described by other workers (2, 4, 7, 9, 10).

We have now further evidence for this third enzyme which we propose to call δ enzyme. This

TABLE III
Effect of various cations on δ proteinase activity

Metal	Azocoll reading in McIlvain buffer	Azocoll reading in Ca borate buffer	Undigested casein after	
			1 hr.	3 hrs.
			%	%
Ca	540	—	18	0
Mg	420	284	20	0
Zn	435	157	100	62
Mn	415	240	25	0
Fe^{++}	390	248	28	0
Co	355	207	28	0
Cu	340	23 (Blank)	100	85
Fe^{+++}	296	158	100	50
Pb	250	131	100	40
Ni	210	24 (Blank)	100	100
Ag	112 (Blank)	41 (Blank)	100	100
Hg	100 (Blank)	122 ?	100	100
None (control)	360	157	25	0
Blank	150		100	100

TABLE IV
Titration of proteinase against Azocoll using different antisera*

Serum	Anti- β	Anti- γ	Anti- δ
2001	100	100	100
2005	350	160	70
2008	370	360	170
2035	230	150	170
8003	260	550	110
8006	180	410	110

* The discrepancies between the anti- γ and anti- δ readings will be obvious and indicate that these two proteinases are antigenically distinct. (Sera were kindly supplied by Dr. C. L. Oakley who calibrated them and who has largely confirmed our results.)

evidence, which will be given in greater detail elsewhere, can be summarized as follows: Tests conducted with a series of six specific antisera calibrated by Oakley and Warrack (7) for anti β (collagenase) and anti γ (cysteine activated proteinase) activity show that the new δ proteinase is immunologically distinct from both β and γ (see Table IV).

Heating of the proteinase preparation to 50° for 30 minutes, lowering of the pH to 4.6, or passage through a column of the ion exchange resin XE64 (Roehm and Haas) results in a loss of $\frac{2}{3}$ or more of the activity towards the synthetic substrates benzoyl arginine amide and arginine ester. This differential inactivation indicates the presence of at least two enzymes in the proteinase preparation. While collagenase is equally thermolabile and sensitive to lowering of pH, losses in activity of proteinase preparations are too great to be accounted for by traces of collagenase present in some of our preparations and inactivation is the same when collagenase-free preparations are subjected to heat and low pH.

We therefore found that at least three proteolytic enzymes are elaborated by *Cl. histolyticum*: a collagenase identical with Oakley's β enzyme, a cysteine activated proteinase, possibly Oakley's γ enzyme, that attacks certain synthetic substrates and resists heat and low pH values and a new more labile proteinase, unaffected by cysteine and similar reducing agents.

SUMMARY

Conditions for the separation of proteolytic enzymes from *Cl. histolyticum* have been established. A proteinase free of collagenase activity and a

powerful collagenase, which, although it still contains other proteinases, is considerably enriched and concentrated with regard to collagen digesting capacity have been isolated. The proteinase preparation has been investigated for the effect of pH, cations and anions on its activity. Its stability has been determined. The main constituent of the proteinase preparation made by the method described is believed to be a new enzyme.

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We are further indebted to Dr. Jerome Gross, Massachusetts General Hospital for the electronmicrophotographs and permission to publish these before his own work is ready for press.

Dr. Harry Goldenberg of the Brooklyn Jewish Hospital very kindly put at our disposal the synthetic substrates benzoyl arginine isopropyl ester, arginine methyl ester, tyrosine ethyl ester and glycine ethyl ester, prepared by him.

The electrophoresis runs and photographs (Figure 1) were made by Dr. Daniel Moore of this institution.

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