Studies on Proteolytic Activity of Mycoplasmas: the Preparation and Properties of Gelatinolytic Enzymes from Strains of *Mycoplasma arthritidis*†

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SUMMARY

Gelatinolytic enzymes secreted by two strains of *Mycoplasma arthritidis* were partly purified by ammonium sulphate and acetone precipitation and separated by chromatography on G-200 Sephadex. Both were of relatively high molecular weight, were active over a broad pH range and displayed substrate specificity. Both released amino groups from gelatin; one released hydroxyproline too. They may represent a new group of proteolytic enzymes since: (i) though specific for collagenous proteins they attacked only denatured, not native, collagen; and (ii) unlike other recognized proteolytic enzymes they did not attack α -casein.

INTRODUCTION

Various proteolytic enzymes have been isolated from micro-organisms, including collagenolytic enzymes from certain strains of clostridia (Mandl, MacLennan & Howes, 1953). Earlier we demonstrated qualitatively that certain strains of mycoplasma produced enzyme(s) capable of preventing dilute gelatin gels from setting at 4 °C (Czekalowski, Hall & Woolcock, 1973). The present communication reports the isolation of two enzymes from each of two strains of *Mycoplasma arthritidis*. Although unequivocal proof of the chemical homogeneity of these preparations has not been obtained, they appear to be sufficiently purified to warrant an investigation of their properties.

METHODS

Cultures of mycoplasmas. *Mycoplasma arthritidis*, strains PG 6 and PG 27, obtained from Dr D. Taylor-Robinson, were grown in liquid growth-medium in 250 ml flat bottles in a humid atmosphere consisting of 5% CO₂ and 95% air (v/v). Growth-medium (200 ml) containing 1% (v/v) 'Difco PPLO serum fraction' (Difco Laboratories Inc., Detroit, Michigan, U.S.A.) was seeded with a 10% (v/v) inoculum of actively growing 4-day-old mycoplasma culture and incubated for 6 days at 37 °C (Czekalowski *et al.* 1973). The organisms were then removed from the culture by centrifugation at 60 000 *g* (Christ-Omega II centrifuge) for 45 min at 4 °C.

Distilled water. Deionized, all-glass-distilled and autoclaved water (pH 6.9) was employed throughout.

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Antibiotics. Penicillin G (Glaxo Laboratories, Greenford, Middlesex) and streptomycin sulphate (Dista Products Ltd, Liverpool) were dispensed separately as aseptic stock solutions of 10 000 u/ml and 10 000 µg/ml, respectively, and stored at -15°C .

Buffers used in the assessment of the effect of pH on the activity of enzymes. For pH values of 7 and above, tris-HCl buffer was used and for pH values below 7 an acetate buffer was employed. Both buffers were prepared in distilled water at 0.05 M.

Fractionation of enzyme preparations. For preliminary fractionation, supernatants of mycoplasma cultures were treated at 15°C with solid ammonium sulphate, Analar grade (British Drug Houses Ltd, Poole, Dorset), to 55 %, 65 % and 100 % saturation (Mandl *et al.* 1953). Each precipitate was removed by centrifugation at 7500 *g* for 1 h at 4°C , washed with an aqueous $(\text{NH}_4)_2\text{SO}_4$ solution of the corresponding saturation and dialysed against distilled water at 4°C . Further separation of the enzymes from non-specific protein was achieved by reprecipitation of these dialysates at 4°C by the stepwise addition of acetone cooled to -20°C (Hall & Gardiner, 1955). Final purification of the enzymes was accomplished on Sephadex G-200 columns (dimensions 2.5×38 cm; flow rate 15 ml/h; elution by downward displacement with 0.05 M-tris-HCl buffer, pH 7.2). The eluant was monitored at 280 nm for its protein content and was collected in 5 ml quantities.

Measurement of gelatinolytic activity. The amount of amino group liberated in a given time was measured by a modification of the method devised by Mandl *et al.* (1953) for the assessment of collagenase activity: 5 ml of 0.05 M-tris-HCl buffer (pH 7.0) containing 0.005 M- CaCl_2 and 20 mg Difco gelatin, 0.5 ml enzyme solution containing between 0.5 and 5 mg of enzyme protein, with the addition of 0.1 ml of a solution of penicillin (1000 u/ml) and 0.1 ml of streptomycin (1000 µg/ml), were incubated at 37°C for periods of up to 36 h, the usual time being 18 h. Samples (0.2 ml) taken at zero time and at the end of the reaction were assayed for amino groups. Controls contained 0.5 ml of 0.85 % (w/v) NaCl in place of enzyme solution.

Ninhydrin reagent was made by mixing equal volumes of a 4 % (v/v) solution of indonetrione hydrate (B.D.H., Analar grade) in methyl cellosolve with 0.4 M-citrate buffer, pH 5, containing 16 mg stannous chloride/ml. A 0.2 ml sample plus 2 ml ninhydrin reagent in a test tube covered with a loose aluminium cap, was placed in a boiling water bath for 20 min and then cooled to room temperature in cold water. Ten millilitres of a 1:1 (v/v) mixture of propan-1-ol and water were added and, after mixing, the mixture was held at room temperature for 15 min before the absorbance was read at 600 nm against distilled water. Leucine was used as standard, and activity was expressed in terms of micrograms leucine equivalent released/h/mg of enzyme protein ($1 \mu\text{g released/h/mg} = 1 \text{ unit/mg}$).

Measurement of general proteolytic activity. Conventional proteolytic activity was measured by the method of Kunitz (1947) using α -casein (B.D.H. Ltd) as substrate. The extent of hydrolysis was assessed by measuring the released amino groups, using the ninhydrin method described above.

Measurement of protein concentration. The method of Lowry, Rosebrough, Farr & Randall (1951) was used for all protein estimations, using 0.5 ml samples and bovine serum albumin (Sigma London Chemical Co. Ltd, Kingston-upon-Thames, Surrey) as standard.

Characterization of the enzymes

After the final stage of purification, attempts were made to characterize both enzymes isolated from supernatants of *Mycoplasma arthritidis* cultures; these are referred to in the results section as the enzymes of peaks 1 and 2, respectively.

The effect of temperature on the activity of the enzymes. Samples of the enzymes eluted in

peaks 1 and 2 from Sephadex G-200 columns, adjusted with distilled water to 1.5 and 10 mg/ml of protein respectively, were heated in a water bath at 60, 80 and 100 °C for 30 min. Their activities were then compared with those of control samples maintained at 4 °C.

Determination of the Michaelis constant. Using the method for measuring gelatinolytic activity, but providing each enzyme with substrate concentrations ranging from 1 to 8 mg/ml, the Michaelis constant was calculated from the reciprocal of the intercept on the $1/[S]$ -axis of Lineweaver-Burk plots of $1/v$ against $1/[S]$.

Estimation of molecular weights of the enzymes. Elution volumes were measured as a basis for the determination of the mol. wt of the most highly purified preparation of each enzyme (Andrews, 1964, 1967; Squire, 1964). Apoferritin, bovine serum albumin, catalase, cytochrome *c* and lactate dehydrogenase were used as markers with established mol. wt. The elution volume was determined for each, its emergence from the column being monitored by measurement of the absorbance at 280 nm. The void volume of the column was assessed with a preparation of blue dextran of mol. wt 2×10^6 (Pharmacia, Uppsala, Sweden) which was completely excluded from the gel. The logarithms of the mol. wt. were then plotted against the retention volumes, i.e. the measured elution volumes minus the void volume of the column.

Evaluation of substrate specificity. The release of hydroxyproline was measured to ascertain whether the enzymes isolated from *Mycoplasma arthritidis* reacted with gelatin itself, or liberated amino groups from other, non-gelatinous but associated, proteins. The reaction mixture for these estimations was identical with that used in the assay of gelatinolytic activity and the samples for investigation were removed at zero time and after 18 h of incubation at 37 °C. These were ultra-filtered under pressure through an Amicon U.M.-10 membrane (Amicon N.V., Oosterhout (N.B.), Holland) which passed all material of mol. wt below 10 000. The filtrate was hydrolysed with an equal volume of 10 N-HCl in a sealed tube maintained at 110 °C for 17 h, the hydrolysate neutralized with 10 N-NaOH, and the hydroxyproline content measured using the automated method of Grant (1964).

RESULTS

Period of incubation. Preliminary experiments showed that enzymatic activity appeared in the supernatant of mycoplasma cultures after 3 days of incubation, increasing over the next 3-day period but not thereafter. For this reason, a 6-day incubation period was accepted as standard. Evaluation of the essential components of the culture medium justified the addition of 1 % of 'Difco PPLO Serum Fraction', which contaminated the medium with far less extraneous protein than did the 20 % horse serum initially employed.

Precipitation with ammonium sulphate. Mandl *et al.* (1953) demonstrated that the majority of their clostridial collagenase activity could be separated into three fractions at 0 to 55, 55 to 65 and 65 to 100 % ammonium sulphate saturation. Similar fractionation of the supernatant from both PG 6 and PG 27 cultures yielded the results presented in Table 1 which records the gelatinolytic activity of these fractions assayed against a substrate prepared from commercial Difco gelatin (Czekalowski *et al.* 1973). Controls consisting of similar fractions prepared from uninoculated culture medium gave low 'blank' values.

The most active fraction isolated from the PG 6 culture medium was that precipitated between 55 and 65 % ammonium sulphate saturation, whereas in the case of PG 27 the 65 to 100 % fraction was almost equally as active. Since little activity was present in the fractions precipitated by 0 to 55 % ammonium sulphate saturation, only the precipitates obtained at the two higher concentrations were further examined.

Table 1. Gelatinase activity of fractions derived from 1000 ml volumes of culture supernatant of two strains of *Mycoplasma arthritidis* by precipitation with increasing concentrations of ammonium sulphate

Activity is expressed in units of gelatinase activity: 1 unit being the number of μg of amino group liberated from gelatin/h.

Strain of <i>Mycoplasma</i>	% saturation with ammonium sulphate		
	0 to 55	55 to 65	65 to 100
PG 6	88.9	400.0	443.1
PG 27	76.38	108.3	387.5
Control	4.2	4.9	11.1

Table 2. The protein content and specific activity of the various acetone fractions from two ammonium sulphate precipitates obtained from each of the two mycoplasma strains

Strain of <i>Mycoplasma</i>	% (NH ₄) ₂ SO ₄ saturation	Acetone concentration							
		0 to 37.5 %		37.5 to 50 %		50 to 60 %		Supernatant at 60 %	
		A	B	A	B	A	B	A	B
PG 6	55 to 65	56	2.6	35	4.1	100	3.0	660	19.3
PG 6	65 to 100	22	4.2	400	0	215	0.9	1125	0
PG 27	55 to 65	60	4.1	27	3.6	17	2.5	515	24.7
PG 27	65 to 100	25	7.5	320	3.8	145	0.5	1025	7

A, Protein concentration (mg/ml); B, specific activity (units/ml). For definition of a gelatinase unit see Table 1.

Fig. 1

Fig. 2

Fig. 1. Fractionation on G-200 Sephadex of 55 to 65 % saturated (NH₄)₂SO₄ precipitate from *Mycoplasma arthritidis* (strain PG 27) culture supernatant. Elution with 0.5 M-tris-HCl buffer, pH 7.2, at a flow rate of 15 ml/h. Curve A: enzyme activity units/ml eluate. Curve B: protein content measured by % transmission at 280 nm.

Fig. 2. Fractionation on G-200 Sephadex of 0 to 37.5 % acetone precipitate obtained from the 65 to 100 % saturated (NH₄)₂SO₄ fraction of *Mycoplasma arthritidis* (strain PG 27) culture supernatant. Curve A: activity of the enzyme in units/ml eluate. Curve B: protein content measured by transmission at 280 nm.

Table 3. Summary of the purification of gelatinase activity by the sequential treatment of 1000 ml supernatant from cultures of *Mycoplasma arthritis* (PG 27) with ammonium sulphate and acetone followed by chromatography on G-200 Sephadex

Preparations	Total protein (mg)	Total activity (units)*	Specific activity (units/mg)
1. Culture supernatant	1207	—†	—†
2. (NH ₄) ₂ SO ₄ ; 55 to 65 % saturation	72.6	78.6	1.08
3. Peak 1 obtained from preparation 2	0.54	13.78	25.52
4. (NH ₄) ₂ SO ₄ ; 65 to 100 % saturation	198.3	325	1.64
5. 0 to 37.5 % acetone precipitate from preparation 4	6.5	283	43.5
6. Peak 1 from preparation 5	0.2	10.8	54.0
7. Peak 2 from preparation 5	2.2	7.27	3.3

* For a definition of a gelatinase unit see Table 1.

† Unmeasurable because of high blank levels.

Fig. 3

Fig. 4

Fig. 3. The time course of release of amino groups from gelatin by the two enzymes isolated from culture supernatants of *Mycoplasma arthritis*, strains PG 27. Curve A: peak 1 (see Fig. 1, 2). Curve B: peak 2 (see Fig. 1, 2).

Fig. 4. The pH response of the two gelatinolytic enzymes isolated from culture supernatants of *Mycoplasma arthritis*, strain PG 27. Specific activity, as defined in Table 1, is recorded in Curve A for peak 1 and in Curve B for peak 2 (see Fig. 1, 2).

Acetone fractionation. Samples (20 to 200 ml) of the 55 to 65 and 65 to 100 % saturated ammonium sulphate fractions, rendered free of salt by dialysing against distilled water at 4 °C, were treated at this temperature with acetone. Little protein was precipitated from either ammonium sulphate fraction until the concentration of acetone had been raised to 37.5 % (v/v). Further precipitates were obtained at 50 % (v/v) and 60 % (v/v) concentration, respectively (Table 2).

Table 4. *Values obtained for K_m from two series of experiments*

Mycoplasma strain	Method of preparation	Series 1	Series 2
PG 6	55 to 65* peak 1	14.3	16.6
	65 to 100* (0-37.5)† peak 1	13.3	18.2
	65 to 100* (0-37.5)† peak 2	33.3	100
PG 27	55 to 65* peak 1	14.3	16.6
	65 to 100* (0-37.5)† peak 1	14.3	20.0
	65 to 100* (0-37.5)† peak 2	33.3	100

* % Saturation with $(\text{NH}_4)_2\text{SO}_4$. † Concentration (v/v) of acetone.

Table 5. *The release of hydroxyproline from gelatin by various preparations of Mycoplasma arthritidis enzymes*

Enzyme preparations	Hydroxyproline mg/100 ml reaction mixture dialysate
PG 6*	1.85
PG 6†	2.40
PG 6‡	—
PG 27*	1.95
PG 27†	1.65
PG 27‡	0.80

* Peak 1 from material precipitated between 55 and 65 % $(\text{NH}_4)_2\text{SO}_4$ saturation.

† Peak 1 from the 0 to 37.5 % acetone fraction of the material precipitated between 65 and 100 % $(\text{NH}_4)_2\text{SO}_4$ saturation.

‡ Peak 2 from the same preparation as †.

The major part of the activity of the 55 to 65 % saturated ammonium sulphate fraction remained in the supernatant from the 60 % (v/v) acetone treatment, whereas appreciable amounts of the 65 to 100 % saturated ammonium sulphate fractions were precipitated at 37.5 % (v/v) acetone concentration. The supernatant remaining after 60 % (v/v) acetone treatment of the 55 to 65 % saturated ammonium sulphate fraction contained 86 % of the total protein. Since little improvement in specific activity could be expected from such treatment of the crude fraction, subsequent chromatographic fractionation was performed on the whole of the 55 to 65 % saturated ammonium sulphate fraction and also on the portion of the 65 to 100 % saturated ammonium sulphate fraction precipitable by acetone at 37.5 % (v/v) concentration (Table 2).

Gel exclusion chromatography. Elution profiles obtained with G-200 Sephadex, were essentially identical for each strain of the organism. Typical examples for PG 27 are shown in Fig. 1 and 2. The elution pattern of the 55 to 65 % saturated ammonium sulphate fraction showed one sharp major and two broader, but minor, protein peaks. The majority of the gelatinase activity was confined to the former with a small amount of activity present in a shoulder or partially separated peak on its trailing edge. The 37.5 % (v/v) acetone precipitate of the 65 to 100 % saturated ammonium sulphate fraction, having a far lower non-specific protein content, showed two fully separated major protein peaks. Again the first of these contained most of the gelatinase activity with, however, an appreciable amount also present in a small peak eluted from the column immediately after the void volume. The elution volume of the peak from the 55 to 65 % saturated ammonium sulphate fraction was 70 ml, being identical with that of the faster moving (and smaller) of the two peaks separated from the acetone fraction of the 65 to 100 % saturated ammonium sulphate precipitate. The second peak of the latter fraction, however, had an elution volume of 124 ml indicating a

Fig. 5. The determination of the molecular weights of the material in peaks 1 and 2. The line relating the logarithm of molecular weight to retention volumes was obtained by the measurement of retention volumes for: A, cytochrome *c*; B, bovine serum albumin (range of values); C, lactic dehydrogenase; D, catalase; E, apoferritin.

lower mol. wt for this component. The results of these purification procedures are summarized in Table 3.

Time course of the reaction with gelatin. The gelatinolytic activity of the enzymes present in peaks 1 and 2, eluted from the Sephadex column and measured by the quantitative release of amino groups, remained undiminished for up to 18 h of incubation, thereafter decreasing considerably (Fig. 3). The specific activity of the various samples of the peak 1 enzyme averaged 2.1 μg amino group released/mg of protein/h, and for the peak 2 enzyme was 0.43 μg amino group released/mg of protein/h.

Characteristics of the enzymes

pH sensitivity. The enzymes were active over a broad pH range (Fig. 4). Below pH 6, enzymes from both peak 1 and peak 2 showed a rapid decrease in activity; on the other hand, although a fall in activity was also observed above pH 8.5, even at pH 9.5 appreciable activity still remained. For both enzymes the existence of two zones of high activity was observed, one at pH 7.0 and the other at pH 8.25.

Thermal stability. Both enzymes from each mycoplasma strain were completely inactivated at 80 °C. In the case of enzymes from PG 27 heated at 60 °C, peaks 1 and 2 fractions retained 25 and 16%, respectively, of the activity present in the control samples maintained at 4 °C, whereas the enzymes from PG 6 were slightly less resistant to that temperature.

Kinetic behaviour. Values for the Michaelis constants of peaks 1 and 2 enzymes were calculated from Lineweaver-Burk plots and are recorded in Table 4. Identical values were determined for the K_m of the enzyme present in peak 1 irrespective of its source (mean value being 15.9), whereas the values of K_m displayed by samples of the peak 2 enzyme, although showing far greater variation, were significantly higher (mean value approximately 66).

Table 6. *The inhibitory activity of increasing concentrations of salts which appear to act solely by reason of their ionic strength, on the gelatinolytic enzymes in peaks 1 and 2*

Ionic strength	% Inhibition					
	Peak 1			Peak 2		
	NaCl	CaCl ₂	MgCl ₂	NaCl	CaCl ₂	MgCl ₂
0.008	4.0	—	—	2.0	—	—
0.012	—	3.8	12.5	—	3.0	27.0
0.04	8.0	—	—	2.5	—	—
0.06	—	4.0	20.0	—	9.0	27.5
0.08	10.0	—	—	8.5	—	—
0.12	—	7.5	24.0	—	11.0	29.0
0.20	24.4	—	—	14.0	—	—
0.30	—	54.0	31.5	—	38.0	30.0
0.40	31.5	—	—	16.0	—	—
0.60	—	—	72.0	—	—	64.5
1.00	48.0	—	—	38.0	—	—
1.50	—	—	79.0	—	—	76.5
2.00	57.5	—	—	82.0	—	—
3.00	—	—	100.0	—	—	100.0

Molecular weights of the isolated fractions. The elution volumes obtained for the enzymes present in peaks 1 and 2 were 70 and 124 ml, respectively. From the graph prepared from the retention volumes of the marker proteins (Fig. 5), the mol. wt of the enzymes from peaks 1 and 2 were deduced to be approximately 468 000 and 85 000 respectively.

Substrate specificity. Having already discovered (Czekalowski *et al.* 1973) that the mycoplasma enzymes do not hydrolyse native collagen, we now observed that neither enzyme acted upon α -casein. While all four preparations of peak 1 liberated hydroxyproline from gelatin concurrently with release of amino groups, only traces of this substance accompanied the release of amino groups by the enzyme(s) present in peak 2 (Table 5).

The effect of ionic strength on gelatinase activity. The activities of both gelatinases were inhibited by increased concentrations of salts (Table 6). The enzyme from peak 1 showed no inhibition until a relatively high sodium chloride concentration (0.1 M, $\mu = 0.2$) was reached, and the enzyme from peak 2 required an even higher concentration (0.5 M, $\mu = 1.0$) to evoke a similar effect. However, even in 1 M-NaCl more than 50% of the activity of both enzymes still remained. Similar results were obtained when the effects of the chlorides of calcium and magnesium were examined. Although both these salts could produce complete inhibition at high concentration, appreciable inhibition was not apparent until ionic strengths of 0.3 and 0.6, respectively, were reached.

DISCUSSION

The most striking feature of these various enzyme preparations is the similarity which exists between the pairs of enzymes identified as peaks 1 and 2, obtained in different ways from both strains of *Mycoplasma arthritidis*. Table 3 summarizes the purification procedures adopted. Their identical behaviour during purification suggests that corresponding enzymes produced by both strains of *Mycoplasma arthritidis* are closely related, thus strengthening their taxonomic status (Czekalowski *et al.* 1973).

The major problem in the purification of the enzymes was the exceptionally high protein

content of the original culture supernatant, even when 'Difco PPLO Serum Fraction' was substituted for whole serum. Electrophoretic examination of peaks 1 and 2 showed relatively little contaminating protein, although this method would not be sensitive enough to reveal the presence of contaminants amounting collectively to less than 5 % of the total.

The various preparations of peak 1 enzyme were of identical mol. wt (Fig. 5), had similar Michaelis constants and, during the degradation of gelatin, released both amino groups and hydroxyproline. The other, i.e. peak 2, enzyme not only differed substantially in mol. wt from that in peak 1 but had a different Michaelis constant and attacked the substrate with the release of amino groups but not of hydroxyproline (Table 5).

Both enzymes displayed high activity in two pH zones, i.e. at pH 7 and 8.25. At one of these pH values the enzymes might be directly activated while the substrate might be favourably dissociated at the other pH.

No specific inhibitory effect on enzyme activity, other than that attributable to ionic strength, was observed for the chlorides of sodium, calcium and magnesium. Moreover, even 1 M-NaCl caused less than 50 % inhibition of both enzymes. Appreciable inhibition by the chlorides of calcium and magnesium only became apparent when their ionic strengths were raised to 0.3 and 0.6, respectively (Table 6).

Since the mol. wt of the 'heavier' enzyme (from peak 1) was not a simple multiple of that of the 'lighter' enzyme (the factor being almost exactly 5.5) the possibility that the peak 1 enzyme was a polymeric form of the enzyme from peak 2 cannot be entertained. This view gains additional support from the fact that the enzymes attacked the gelatin molecule at quite distinct sites.

Collagen and gelatin molecules consist in the main of helical structures which contain glycine and the imino acids, proline and hydroxyproline, in appreciable amounts and in defined proportions. The telopeptides of these molecules are devoid of hydroxyproline and it is through these regions that cross-linkages are formed between the adjacent chains of the substrate. It may be inferred from the differences in the end products of the reactions of the two enzymes that, whereas the enzyme present in peak 1 breaks linkages within the helical, hydroxyproline-rich portion of the molecule, that in peak 2 attacks bonds which lie within the telopeptide regions.

Czekalowski *et al.* (1973) were unable to demonstrate any attack by mycoplasma enzymes on various forms of undenatured collagen, viz. insoluble collagen from achilles tendon and soluble collagen extracted from skin by neutral salt or dilute acid. Commercial hide powder, however, was slightly affected, but this can be attributed to the rather drastic treatment, including alkaline extraction, applied during its preparation. Only a limited number of specific enzymes, i.e. collagenases, digest native collagen. On the other hand, gelatin, a denatured derivative of collagen, can be further degraded by proteolytic enzymes such as trypsin and pepsin, which are able to break peptide linkages in a number of soluble proteins.

However, neither of our enzymes showed general proteolytic activity and it would appear that they are similar in their substrate specificity, requiring denaturation of the collagenous substrate before they can react with it.

Mandl *et al.* (1953) have shown that calcium ions in relatively low concentrations (5 mM) are required for the optimum rate of collagenase activity with the natural substrate. The failure of calcium ions to activate gelatinolytic enzymes, supports the evidence already provided by Czekalowski *et al.* (1973) that both enzymes produced by *Mycoplasma arthritidis* differ fundamentally from collagenase.

The above findings suggest that both of the mycoplasma enzymes may be representative of a new group of proteolytic enzymes which, though specific for collagenous proteins, are

unable to attack collagen in its native form, requiring its denaturation at least to the degree that would result in its gelatinization. Also, unlike other recognized proteolytic enzymes, they do not attack α -casein which is a typical soluble, denatured, non-collagenous protein.

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