Mycoplasma-induced BALB/c 3T3 collagenase is a mammalian enzyme

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A collagenase previously reported to accumulate in the medium of cultures of BALB/c 3T3 cells on infection with *Mycoplasma orale* [Kluve, Merrick, Stanbridge & Gershman (1981) *Nature (London)* **292**, 855–857] was partially purified and characterized. With regard to purification properties, activation, sensitivity to inhibitors and relative molecular mass the enzyme was similar to previously reported vertebrate collagenases, but could not be unequivocally distinguished from bacterial collagenases. With regard to substrate-specificity and reaction products, however, the collagenase was typical of vertebrate collagenases and distinct from bacterial collagenases. Specifically, the enzyme displayed a preference for type III collagen and type I collagen, a somewhat decreased ability to degrade type II collagen, and a very limited ability to degrade type IV collagen. The initial products of the action of the collagenase on type I collagen were characterized as fragments one-quarter and three-quarters of the length of the intact collagen molecule. Because the properties of the collagenase produced by cultures of mycoplasma-infected BALB/c 3T3 cells are those of a mammalian-type (vertebrate-type) enzyme, we have concluded that the collagenase is a product of the mouse (BALB/c 3T3) genome, and is not produced by the mycoplasma. Therefore it appears that infection of BALB/c 3T3 mouse fibroblasts with *Mycoplasma orale* induces the mouse cells to produce and secrete collagenase.

Vertebrate collagenase is a highly specific proteinase that initiates collagen degradation. In contrast with bacterial collagenases, the animal enzyme makes a single scission in each of the three polypeptide chains of native tropocollagen. This cleavage produces two distinct fragments, termed TC^A and TC^B, representing three-quarters and one-quarter of the collagen molecule respectively. The enzyme is believed to play an important role in the physiological remodelling of tissues during growth, development and repair. In addition, abnormally high collagenase activity may contribute to pathological tissue destruction in rheumatoid arthritis and in periodontal disease, and may permit malignant invasiveness and metastasis (reviewed by Cassell & Cole, 1981).

We have already demonstrated that the addition of pure mycoplasmas to cultures of BALB/c 3T3 cells results in increased accumulation of extracellular collagenase activity (Kluve *et al.*, 1981). In

order to determine the genomic origin of the collagenase produced by mycoplasma-infected BALB/c 3T3 cells, we have compared the properties of the collagenase isolated from cultures of BALB/c 3T3 cells with the published properties of collagenases purified from both bacterial and vertebrate sources. To permit a clear characterization, partial purification of the collagenase was effected.

Methods

Cell culture

BALB/c 3T3 cells were originally derived from the A31 clone isolated by Aaronson & Todaro (1968). The cells were grown in Eagle's Minimum Essential Medium (GIBCO, Grand Island, NY, U.S.A.) with 4-fold increased concentrations of vitamins and amino acids in Earle's salt solution (GIBCO) (medium MEM 4×). This medium was supplemented with foetal-calf serum, penicillin, streptomycin and L-glutamine to final concentrations of 10%, 100 units/ml, 100 µg/ml and 2 mM respectively. Cells were cultured at 37°C in a humidified atmosphere of CO₂/air (1:19) and were

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used between passages 13 and 18. A culture of BALB/c 3T3 cells was infected with a sample of *Mycoplasma orale*. These cells were grown to confluence and subcultured repeatedly until a stable degree of infection was established and a uniformly high collagenase activity was measured.

Collagen substrate

Neutral salt-soluble collagen was extracted from guinea-pig skin in 0.5M-NaCl and purified by a published procedure (Gross, 1958). Purified collagen was radiolabelled by a modification of a reductive methylation procedure developed for non-collagenous proteins (Jentoft & Dearborn, 1979). Collagen at 4 mg/ml in 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 8.0, containing 20 mM-NaCl was treated simultaneously with 0.2 mM-[14 C]formaldehyde (specific radioactivity 56 mCi/mmol) and 2 mM-sodium cyanoborohydride for 24 h on ice. The reaction was terminated by dialysing the collagen solution against 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.2 M-NaCl and 0.05% NaN₃. After a single cycle of gelation/solubilization, the collagen solution was made 10 mM with respect to CaCl₂. The specific radioactivity of the collagen prepared in this manner was 667 c.p.m./ μ g.

Assay of collagenase activity

Collagenase activity was assayed by measuring the release of soluble radioactive peptides from a thermally reconstituted gel of [14 C]methylated native collagen fibrils (Nagai *et al.*, 1966). A typical reaction mixture contained 36 μ g of collagen in a 10 μ l volume and 10–100 μ l of collagenase preparation in 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-CaCl₂, 50 mM-NaCl and 0.05% NaN₃ (collagenase buffer). Enzyme preparations were activated by treatment with 1 mg of trypsin/ml of collagenase preparation, added in 10 μ l of collagenase buffer for 45 min at room temperature, then a molar equivalent of soya-bean trypsin inhibitor in 20 μ l of collagenase buffer. Alternatively, these samples were activated by overnight dialysis at 4°C against 1 mM-aminophenylmercuric acetate in collagenase buffer. Studies in which time, temperature and concentration of aminophenylmercuric acetate were varied indicated that these conditions yielded maximal collagenase activity.

Other procedures

The molecular weight of collagenase was estimated by gel filtration at 4°C on a column (1.4 cm $\times$ 173 cm) of Sephadex G-100. The column was calibrated with the protein standards bovine serum albumin, ovalbumin, soya-bean trypsin inhibitor and myoglobin, having respective *M_r* values of 67 000, 43 000, 20 100 and 17 000. The void and included

volumes were determined with solutions of Blue Dextran and [14 C]leucine respectively.

Electrophoresis was performed in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulphate by following the method of Laemmli (1970) except that one-quarter the concentration of bis-acrylamide was used. This decreased concentration of cross-linker gave gels roughly equivalent to 6–7% gels in terms of resolvable molecular-weight range.

Results

Purification of collagenase

Medium was collected every 4 days over a 20-day period from mycoplasma-infected confluent cultures of BALB/c 3T3 cells growing in medium MEM 4 $\times$ containing 10% foetal-calf serum. The growth conditions and collection schedule had previously been shown to maximize the accumulation of collagenase activity in the medium (Kluge *et al.*, 1981). Collagenase activity in 14.45 litres of culture medium was concentrated and initially fractionated by precipitation at 40–60% saturation with (NH₄)₂SO₄. At this stage of purification most of the collagenase activity was latent; it required preincubation with trypsin or aminophenylmercuric acetate to demonstrate maximum activity.

The total protein present and the specific activity of the collagenase for various stages of the purification procedure are given in Table 1. The enzyme could not be assayed accurately in the starting material, raw culture medium, and therefore fold increases in specific activity have not been calculated.

The latent collagenase preparation was dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing 0.05% NaN₃ and 10% (v/v) glycerol. After clarification by centrifugation, it was applied to a column

Table 1. Purification of BALB/c 3T3 collagenase
For full experimental details see the text.

Purification procedure	Total protein (mg)	Specific activity (μ g degraded/min per mg)
Tissue-culture medium (14.45 litres)		
(NH ₄) ₂ SO ₄ fractionation (40–60% saturation)	10 380	3.3
Phosphocellulose (batch elution)	748	2.0
QAE-Sephadex A-50	189	2.4
Phosphocellulose* (gradient elution)	12.4	3.0
Sephadex G-100*	1.7	15.0

* Collagenase activity bound by phosphocellulose.

(5 cm × 39 cm) of phosphocellulose (Whatman P-11), equilibrated with the same buffer. The bound protein was eluted from the phosphocellulose in a batchwise manner with equilibration buffer containing 1 M-(NH₄)₂SO₄. Fractions were collected into tubes containing enough CaCl₂ to give a concentration of 10 mM. Protein eluted by 1 M-(NH₄)₂SO₄ was concentrated by using Aquacide (Calbiochem) and dialysed against collagenase buffer containing 10% glycerol before being assayed. This preparation exhibited maximum collagenase activity without trypsin treatment, indicating that the enzyme had been eluted from phosphocellulose in active form. No collagenase activity could be detected in the break-through fractions, even after incubation with trypsin over a concentration range of 0–3 mg/ml or after dialysis against 1 mM-aminophenylmercuric acetate.

The collagenase preparation was then subjected to chromatography on a column (5 cm × 19 cm) of QAE-Sephadex A-50 in a solution of 20 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-CaCl₂ and 10% glycerol and was eluted with a 3-litre gradient of 0.02–0.4 M-NaCl in the same buffer. Collagenase was tightly bound by this strong anion-exchanger and was eluted at 0.22 M-NaCl. The sample was concentrated by using Aquacide and applied to a column (2.5 cm × 23 cm) of phosphocellulose, after equilibration of both in 50 mM-Tris/HCl buffer,

pH 7.5, containing 0.05% NaN₃ and 10% glycerol. The column was eluted with a 1-litre linear gradient of 0.0–0.2 M-NaCl in equilibration buffer. Fig. 1 shows that a major portion of the collagenase activity bound to the phosphocellulose and was eluted when the NaCl concentration was increased. A smaller but substantial amount of collagenase activity failed to bind to the column. This activity also failed to bind to a fresh column of phosphocellulose equilibrated with a lower-ionic-strength buffer, eliminating the possibility that the previous column had been overloaded with protein.

The two fractions of activity were individually concentrated by using Aquacide and dialysed against 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-CaCl₂, 0.1 M-NaCl, 0.05% NaN₃ and 10% glycerol. Final purification was achieved by fractionation of both preparations on a column (1.4 cm × 173 cm) of Sephadex G-100 equilibrated with the same buffer. The collagenase that had bound to phosphocellulose was eluted as a symmetrical peak with maximum activity in fraction 68, which corresponded to an *M_r* value of 61 000. Gel filtration of the sample that had not bound to phosphocellulose yielded a profile in which peak activity was present in fraction 74, corresponding to an *M_r* value of 46 500. Although this material was not further characterized in detail, its catalytic properties appeared, in preliminary experiments, to

Fig. 1. Phosphocellulose chromatography of BALB/c 3T3-cell collagenase (gradient elution)

The collagenase preparation from QAE-Sephadex A-50 chromatography, containing 189 mg of protein in 81 ml, was applied to a column (2.5 cm × 23 cm) of phosphocellulose in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.05% NaN₃ and 10% glycerol. Bound protein was eluted with a 1-litre linear gradient of 0–200 mM-NaCl in the same buffer. A flow rate of 38 ml/h was maintained, and 10 ml fractions were collected. Fractions were monitored for protein by absorbance at 280 nm (O) and NaCl concentration (—•—). Samples (50 µl) were assayed for collagenase activity (c.p.m. released into supernatant, ●). For full experimental details see the text.

be identical with those of the bulk of the collagenase activity.

The enzyme activity that had bound to phosphocellulose and had been eluted from Sephadex G-100 with a peak in fraction 68 was shown to cleave native collagen into three-quarter-length and one-quarter-length fragments (see below), thus identifying it as a true vertebrate collagenase. The final yield of this partially purified collagenase was 1.7 mg. The preparation had a specific activity of 15 μ g of collagen degraded/min per mg of protein. This preparation contained no detectable collagenase inhibitors, as tested by mixing experiments, and was free of other proteinase activities, as judged by failure to digest radiolabelled ovalbumin and bovine serum albumin.

Kinetic and inhibitor properties

The pH optimum of the partially purified collagenase was determined to be 7.5–8.0, with a broad range of 80% maximum activity between pH 6.5 and 9.0. This preparation of collagenase digested collagen in a linear fashion with time, and its activity could not be further enhanced by treatment with either trypsin or aminophenylmercuric acetate.

The sensitivity of partially purified collagenase to inhibition by various compounds was tested. The results obtained, shown in Table 2, are again consistent with the properties of other vertebrate collagenases, although it must be recognized that bacterial collagenases would be expected to behave similarly. The enzyme was completely inhibited by 2.5 mM-EGTA, suggesting that Ca^{2+} is required for activity, as it is for all collagenases studied to date. Lowering the Ca^{2+} concentration from 10 mM (normally present in our assay buffer) to 1.25 mM caused only minimal (8%) decrease in activity; however, the collagenase appeared to be more susceptible to inhibition by other agents. For example, cysteine (10 mM) or dithiothreitol (1 mM), in the presence of 1.25 mM- Ca^{2+} , inhibited collagenase activity by 46% in each case, whereas in 10 mM- Ca^{2+} these compounds were not significantly inhibitory. At a very high concentration, namely 100 mM, cysteine produced greater than 90% inhibition even in the presence of 10 mM- Ca^{2+} . The presence of 1,10-*o*-phenanthroline (1 mM) decreased the activity by more than 90%. This is consistent with the idea that collagenases are zinc metallo-enzymes (Berman & Manabe, 1973). *N*-Ethylmaleimide (10 mM) had little or no effect on collagenase activity in the presence of 10 mM- or 1.25 mM- Ca^{2+} , suggesting that free thiol groups are not important in retaining an active conformation. The collagenase was insensitive to phenylmethylsulphonyl fluoride (2 mM) and therefore is not a serine proteinase. In agreement with reports that β_1 -anticollagenase and the potent non-specific inhibitor α_2 -macroglobulin

Table 2. *Inhibition of BALB/c 3T3-cell collagenase activity by various inhibitors*

Reagents were diluted in collagenase buffer or collagenase buffer minus CaCl_2 ; serum was dialysed against and diluted with collagenase buffer. Each reaction mixture contained 36 μ g of [^{14}C]methylated collagen, 44 μ g of collagenase from phosphocellulose chromatography (batch elution) and 175 μ l of buffer containing the various inhibitors. For full experimental details see the text.

Reagent added	Final concn. reagent in assay medium (mM)	Concn. of Ca^{2+} in assay medium (mM)	Inhibition (% of control activity)
None	—	10	—
None	—	1.25	8
EGTA	5	1.25	94
Cysteine	10	10	2
Cysteine	10	1.25	46
Cysteine	100	10	91
Cysteine	100	1.25	89
Dithiothreitol	1	10	0
Dithiothreitol	1	1.25	46
<i>N</i> -Ethyl- maleimide	10	10	0
<i>N</i> -Ethyl- maleimide	10	1.25	22
Phenylmethane- sulphonyl fluoride	2	10	7

are present in serum, we found that foetal-calf serum at 83%, 8.3% and 0.83% (final concentrations in the assay mixture) inhibited activity by 93%, 37% and 11% respectively. When serum-containing medium removed from cultures of BALB/c 3T3 cells was fractionated on phosphocellulose columns, as described above, the inhibitory activity failed to bind to the ion-exchanger, whereas the collagenolytic activity bound and was eluted when the salt concentration was increased. The eluted collagenase was fully active, suggesting that the latency observed in less-purified fractions was due to the presence of one or more inhibitors. When the break-through fractions from phosphocellulose were mixed with the eluted active collagenase, the enzyme was totally inhibited; however, when the same break-through fraction was first dialysed against 1 mM-aminophenylmercuric acetate it was no longer inhibitory.

Substrate-specificity

Activity towards types I, II, III and IV collagens was tested, and the results are shown in Fig. 2. Assays were performed, as previously described (Liotta *et al.*, 1979), in a similar manner to the standard collagenase assay, except that collagen from other sources was used. The preparation of

Fig. 2. Relative activity of BALB/c 3T3-cell collagenase against types I, II, III and IV collagens

Samples of collagenase that had bound to phosphocellulose (gradient elution column) and that contained the indicated amounts of protein were assayed by the method of Liotta *et al.* (1979) for their ability to degrade various collagens. Control assays were performed with 0.1% bacterial collagenase to achieve the maximum degradation value. The substrates were type I collagen prepared from guinea-pig skin (Nagai *et al.*, 1966) ($\blacktriangle$), type II collagen isolated from a human chondrosarcoma serially transplanted in nude mice ($\blacksquare$), type III collagen purified from foetal-calf skin ($\bullet$), and type IV collagen prepared from organ cultures of EHS sarcoma (Liotta *et al.*, 1979) ($\blacktriangledown$). For full experimental details see the text.

these substrates has been described (Liotta *et al.*, 1979). The collagen used in this experiment was radiolabelled by using NaB^3H_4 (Means & Feeney, 1968). The maximal digestion possible was estimated from parallel gels that were treated with bacterial collagenase from *Clostridium histolyticum*. Release of peptides from radiolabelled gels of types I–IV collagens indicated that the BALB/c 3T3-cell enzyme degraded type III collagen most rapidly, type I collagen somewhat less well, and type II collagen even less efficiently. Minimal degradation of basement-membrane collagen type IV was observed. All four of the collagens tested were degraded equally well by bacterial collagenase, in direct contrast with the behaviour of these substrates towards the BALB/c 3T3-cell enzyme and other vertebrate collagenases (Liotta *et al.*, 1979; Bornstein & Sage, 1980).

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and radioautography of [^{14}C]methylated collagen after incubation with BALB/c 3T3-cell collagenase

Lane (a): $24\mu\text{g}$ (16000 c.p.m.) of [^{14}C]methylated collagen before incubation with collagenase. Lane (b): $24\mu\text{g}$ (16000 c.p.m.) of [^{14}C]methylated collagen incubated with the most highly purified collagenase preparation. The reaction mixture contained $12\mu\text{g}$ of collagenase and $24\mu\text{g}$ of collagen in 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM- CaCl_2 , 50 mM-NaCl, 1 mM-phenylmethanesulphonyl fluoride, 0.04% NaN_3 and 6.25% glycerol in a final volume of $32\mu\text{l}$. After 24 h at 23°C , the reaction was terminated by the addition of ice-cold trichloroacetic acid (final concn. 10%, w/v). The protein that was precipitated was subjected to electrophoresis in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate in accordance with the method of Laemmli (1970). After electrophoresis, the gel was dried and radioautographed by using Kodak X-Omat film. The radioautographs were then scanned, with white light in a Transdyne General Corp. model 2500/2150 densitometer. The scan of the TC^B -fragment region of the gel was made from a radioautograph that had been exposed twice as long as the scan of the higher-molecular-weight regions of the gel.

Collagen cleavage products

Collagenase was incubated with type I collagen at 23°C for 24 h. The products of this reaction were examined by polyacrylamide-gel electrophoresis and radioautography. Densitometric scans of these radioautographs are presented in Fig. 3. The loss of radioactivity from the bands corresponding to α_1 - and α_2 -chains was accompanied by the appearance of radioactivity in two major bands with mobilities

characteristic of TC^A fragments. Radioactivity in the region of the β -polypeptides, the cross-linked dimer of α -chains, had also disappeared by 24 h, at which time radioactive bands representing β -TC^A fragments had appeared. The TC^B fragments, which were detected by overexposing the radioautographs relative to the TC^A fragments, migrated in positions consistent with a size equal to one-quarter of the length of the intact molecule. It appears that the α_1 -TC^A fragment is more stable to further proteolytic digestion than are the α_2 -TC^A and TC^B fragments, as judged by their more rapid degradation.

Discussion

Infection of cultures of BALB/c 3T3 cells with *Mycoplasma orale* results in the accumulation of extracellular collagenase in the culture medium (Kluge *et al.*, 1981). We have previously determined, confirming reports in the literature, that pure cultures of *Mycoplasma orale* do not produce collagenase. Similarly, uninfected 3T3 cells produce only small amounts of collagenase. The appearance of collagenase in cultures of 3T3 cells infected by *M. orale* may therefore represent a true induction in which the mammalian cells produce and secrete this enzyme in response to the presence of the bacterium. Alternatively, the collagenase produced might be the product of the bacterial genome expressed only when the mycoplasmas are grown in the presence of eukaryotic cells. In order to determine the genomic origin of the collagenase, we have taken advantage of differences in the properties of vertebrate and bacterial collagenases by comparing the properties of the mycoplasma-infected BALB/c 3T3-cell enzyme with those of other well-characterized collagenases.

The results that we obtained are consistent with the collagenase being of mammalian rather than bacterial origin, and also indicate that the enzyme is not one of the known lysosomal cathepsins. The enzyme maximally degrades collagen at physiological temperatures and at a pH between 7.5 and 8.0. This trait distinguishes it from other proteinases such as lysosomal cathepsin B₁, which can degrade collagen but only at pH 4.0–4.5 (Burleigh *et al.*, 1974), and eliminates the possibility that mycoplasma infection results in cell death and concomitant appearance of this lysosomal proteinase into the medium.

The collagenase displayed a variety of properties consistent with those of previously studied vertebrate collagenases: the collagenase was latent in raw culture medium or in medium that had been fractionated only by (NH₄)₂SO₄ precipitation. Pretreatment with trypsin at room temperature, dialysis against 1 mM-aminophenylmercuric acetate, chromatography on phosphocellulose, extended incu-

bation at 37°C or prolonged storage at –20°C all activated the enzyme. The latency of vertebrate collagenases and their susceptibility to activation have been well documented (Murphy & Sellers, 1980). The *M_r* value of our purified collagenase is approx. 61 000, as determined by gel filtration, a value consistent with several reports in the literature (e.g. Woolley *et al.*, 1975b, 1978; Stricklin *et al.*, 1977). Studies with a variety of inhibitors were also consistent with previous reports. In addition, the substrate-specificity and cleavage products of the reaction of our purified enzyme with collagen make it clear that the collagenase produced by mycoplasma-infected BALB/c 3T3 cells is a vertebrate-type enzyme and quite distinct from the bacterial enzymes reported to date.

The enzyme produced by infected cultures of BALB/c 3T3 cells degraded types I and III collagens more efficiently than type II collagen and demonstrated negligible activity towards type IV collagen. This is identical with the substrate-specificity exhibited by rheumatoid synovial collagenase (Woolley *et al.*, 1975a). The inability to degrade basement-membrane collagen type IV appears to be a property common to the vertebrate collagenases, which act on the interstitial collagens, types I, II and III (Liotta *et al.*, 1979; Woolley *et al.*, 1978). In contrast, bacterial collagenases do not display preference for collagen types (Liotta *et al.*, 1979).

Perhaps most significantly, the reaction products that we observed are diagnostic for mammalian collagenases: the enzyme cleaved type I collagen into two discrete fragments, three-quarters and one-quarter of the length of the molecule. This unique scission is a distinctive feature of all vertebrate collagenases. Bacterial collagenases, in direct contrast, cleave collagen at multiple sites, generating many small peptides (Stark & Kuhn, 1968).

On the basis of the properties described above, we have concluded that infection of BALB/c 3T3 cells by *M. orale* results in the induction of synthesis and secretion of the product of the mouse collagenase gene. This induction has profound implications both for the normal regulation of collagenase activity by direct cell–cell contact and in the inappropriate expression of this gene in a variety of disease states.

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