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CHARACTERIZATION AND SERUM INHIBITION OF NEUTRAL COLLAGENASE FROM CULTURED DOG GINGIVAL TISSUE

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Summary

1. Explants of dog gingiva, maintained in culture for 9 days in the absence of serum, released a collagenase (EC 3.4.24.3) into the medium. The yield of active enzyme reached a maximum after 5—8 days with concomitant release of collagen degradation products from the explants.

2. The enzyme attacked undenatured collagen in solution at 25° C resulting in a 58% loss of specific viscosity and producing the two characteristic products $TC_A(3/4)$ and $TC_B(1/4)$. Electron microscopy of segment-long-spacing crystallites of these reaction products showed the cleavage locus of the collagen molecule at interband 40.

3. Optimal enzyme activity was observed over the pH range 7.5—8.5 and a molecular weight of approximately 35 000 was derived from gel filtration studies. EDTA, 1,10-phenanthroline, cysteine and dithiothreitol all inhibited collagenase activity. Proteoglycan derived from porcine and human cartilage did not inhibit the enzyme.

4. The enzyme was inhibited by the dog serum proteins α_2-macroglobulin and a smaller component of molecular weight approximately 40 000. This smaller component was purified by column chromatography utilising Sephadex G-200, DEAE A-50, and G-100 (superfine grade). Agarose electrophoresis of the purified component showed it to represent a β-serum protein. α_1-Antitrypsin did not inhibit the enzyme.

5. The physiological importance of the natural serum inhibitors and gingival collagenase are discussed in relation to latent enzyme and periodontal disease.

Introduction

Several studies have suggested that neutral collagenase (EC 3.4.24.3) is involved in the breakdown and loss of gingival collagen which accompanies

plaque-induced inflammatory periodontal disease [1—3]. Collagenase has been identified in crevicular fluid from inflamed gingiva [4,5] and its activity was found to increase with the severity of disease [6]. Since the aetiology, pathology and natural history of periodontal disease in dogs closely resembles that in humans we have undertaken the characterization of dog gingival collagenase.

The regulatory meachanism of collagenase activity in vivo is probably complex since recent reports suggest that gingival tissue produces a latent enzyme [7—9]. However, in order to understand the data on latent collagenase it is essential to know what enzyme inhibitors are present in the explanted tissue initially, and also if other inhibitors are released by the cells during culture. As the gingival tissue fragments are known to contain serum proteins when explanted in culture, we have examined whole serum to determine which anti-proteinases may inhibit gingival collagenase.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium with HEPES buffer and 0.85 g/l $NaHCO_3$ was purchased from Flow Laboratories. Agarose film plates were supplied by Corning ACI. Preparations of cartilage proteoglycans were kindly provided by Drs. T. Hardingham, C. McDevitt and Helen Muir of the Kennedy Institute of Rheumatology, London. Other reagents and chemicals were as described previously [10].

Tissue culture. Small amounts of healthy gingival tissue, essentially free of inflammation, were taken from anaesthetised Beagle dogs under aseptic conditions. The tissue was cut into small pieces measuring approximately 3 mm in largest dimension and washed with three changes of Dulbecco's modified Eagle's medium supplemented with antibiotics as described previously [10]. The gingival fragments were transferred to plastic culture flasks containing culture medium and incubated at 37°C for 8 or 9 days. The medium was changed at 24-h intervals and stored at −20°C after adjustment to 20 mM Tris buffer (pH 8.0) and 2 mM $CaCl_2$. Culture media containing collagenase activity were concentrated by ultrafiltration at 4°C on Amicon PM-10 membranes and this was used as the enzyme preparation in all the experiments described.

Collagenase assay. Collagenolytic activity was assayed by measuring the release of soluble radioactive products from a pellet of thermally reconstituted [^{14}C]glycine-labelled collagen fibrils as described previously [10]. Each assay contained approximately 60 μg collagen and 2500 cpm of ^{14}C-radioactivity.

Proteinase assays. Trypsin, papain and thermolysin were assayed using casein and azocasein substrates as described previously [11]. With each series of assays, control incubations containing twice the amount of enzyme were included to ensure that reactions were terminated at a point where they were still linear.

Glucose, hydroxyproline and protein measurement. Glucose concentrations of the culture medium were measured by the method of Hultman [12] using *o*-toluidine reagent. Hydroxyproline was measured using a technique modified from that described by Woessner [13] with internal hydroxyproline standards. Protein was determined by the Folin method of Lowry et al. [14] with crys-

talline bovine serum albumin as a standard.

Disc gel electrophoresis. Electrophoresis in 5% polyacrylamide gels containing sodium dodecyl sulphate [15] was used to examine collagen degradation products as described previously [10].

Molecular weight estimation. The molecular weights of the enzyme and serum inhibitor were estimated by gel filtration at 4°C in a column (35 × 1.5 cm) of Sephadex G-100 superfine grade. The eluting buffer was 20 mM Tris · HCl, pH 8.0, containing 0.17 M NaCl and 10 mM $CaCl_2$, and 1-ml fractions were collected at a flow rate of 5 ml/h. The column was calibrated with the protein standards transferrin, ovalbumin, carbonic anhydrase and myoglobin. The void-volume and salt-exclusion values were determined with solutions of Blue Dextran and potassium ferricyanide respectively. K_{av} was calculated from $(V_e - V_0)/(V_t - V_0)$ and plotted against log molecular weight of the protein standards. The column eluate was monitored at 280 nm and the ^{14}C-labelled collagen fibril assay was used to determine the presence of collagenase inhibitor.

Electron microscopy. Segment-long-spacing crystallites of collagen and the products resulting from collagenase cleavage were prepared as previously described [10]. Crystallites stained with 0.1% phosphotungstic acid and 0.01% uranyl acetate were examined with an AEI 801A electron microscope operated at 80 kV.

Serum protein fractionation. Beagle serum was fractionated by gel filtration at 4°C in a column (90 × 2 cm) of Sephadex G-200 using an eluting buffer of 20 mM Tris · HCl (pH 8.0) containing 0.17 M NaCl and 10 mM $CaCl_2$. Absorbance at 280 nm was monitored and the 2-ml fractions were examined for inhibitory activity against gingival collagenase in the collagen fibril assay, and against trypsin using the casein assay method [11].

The small molecular weight fractions (albumin peak) were pooled, concentrated, dialysed against 20 mM Tris · HCl buffer (pH 8.1) containing 10 mM $CaCl_2$ and applied to a column of DEAE-Sephadex, A.50. The bound proteins were eluted with increasing concentrations of NaCl dissolved in the equlibrating buffer and the eluant fractions were assayed for inhibitory activity against collagenase and trypsin.

Agarose electrophoresis. The small molecular weight serum inhibitor was examined by agarose electrophoresis using a 50 mM barbital buffer (pH 8.0) for 1 h at 3 mA [11]. The protein bands were stained with Coomassie Brilliant Blue R250 and the serum protein classification was deduced from previous studies [16].

Results

Tissue culture of gingival explants

Explants were cultured for 9 days and the medium collected from each day of culture was examined for collagenolytic activity, hydroxyproline and glucose content (Fig. 1). Collagenase activity measured by the [^{14}C]collagen fibril assay was shown to appear in the culture medium after day 2, increasing to maximal values for days 6—8 of culture. A concomitant increase in the

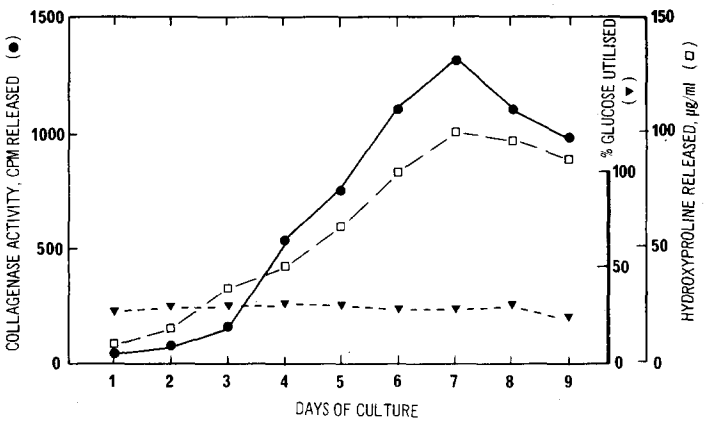

Fig. 1. Release of collagenolytic activity and hydroxyproline from dog gingival explants in tissue culture. Culture medium harvested on successive days of culture was assayed for collagenase activity (●) hydroxyproline (□) and glucose (▼) content as described in Methods.

release of hydroxyproline peptides into the medium was observed over the same time course, indicating collagen breakdown in the explants. Glucose uptake by the explants, as judged by measurement of the glucose concentration remaining in each day's medium, suggested that the explants retained good viability throughout the period of culture.

Viscometric studies and pH optimum

Viscometric studies at 25°C showed that the addition of enzyme to a collagen solution resulted in a 58% loss of specific viscosity. The pH-dependence of the enzyme was assessed with the [^{14}C]collagen fibril assay using Tris/maleate and Tris · HCl buffers [10]. Optimal enzyme activity was observed over a neutral pH range of 7.5—8.5 with no activity below pH 5.0.

Disc gel electrophoresis

Electrophoresis in 5% polyacrylamide gels containing sodium dodecyl sulphate was used to examine the products of the reaction between enzyme and collagen in solution at 25°C. Fig. 2 shows that two products were formed which represented approximately 3/4- and 1/4-length fragments of the collagen molecule. These are labelled TC_A and TC_B respectively. The two protein bands comprising each fragment represent the α_1 and α_2 peptides which have different electrophoretic mobilities as shown in the control gel A.

Segment-long-spacing crystallites

The cleavage products resulting from the exposure of collagen in solution to the gingival collagenase at 25°C were further examined by electron microscopy of segment-long-spacing (SLS) crystallites. Fig. 3 shows crystallites of the intact collagen molecule TC and the TC_A(3/4) fragment produced by collagenase attack. Measurement of these crystallites showed the TC_A fragment to be 75% of the length of the collagen molecule from the amino-terminal end. Alignment of the banding patterns with a clearly defined TC crystallite indicated the cleavage locus was located between bands 40 and 41 according to the nomenclature

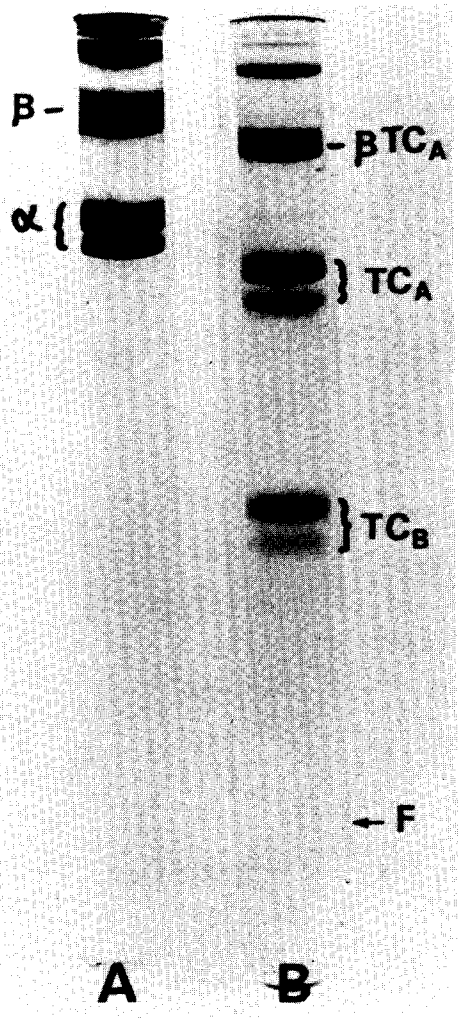

Fig. 2. Disc-gel electrophoresis of the reaction products of dog gingival collagenase and collagen in solution at 25° C. The control sample without enzyme (A) and the reaction products obtained with enzyme (B) were equivalent to 50 μg of collagen. Each was examined by electrophoresis in 5% polyacrylamide gels containing sodium dodecyl sulphate, F, buffer front.

of Bruns and Gross [18]. SLS crystallites of the $TC_B(1/4)$ fragments were also observed and the measurement and banding patterns of these (not shown) conformed with the missing portion of the crystallite shown in Fig. 3(B).

Molecular weight estimation

Concentrated gingival collagenase was subjected to gel filtration on a column of Sephadex G-100 superfine grade, previously calibrated with protein standards of known molecular weight. The enzyme activity was eluted as a single peak corresponding to molecular weight of approximately 35 000.

Enzyme-inhibition studies

Table I shows the effect of various agents on gingival collagenase activity.

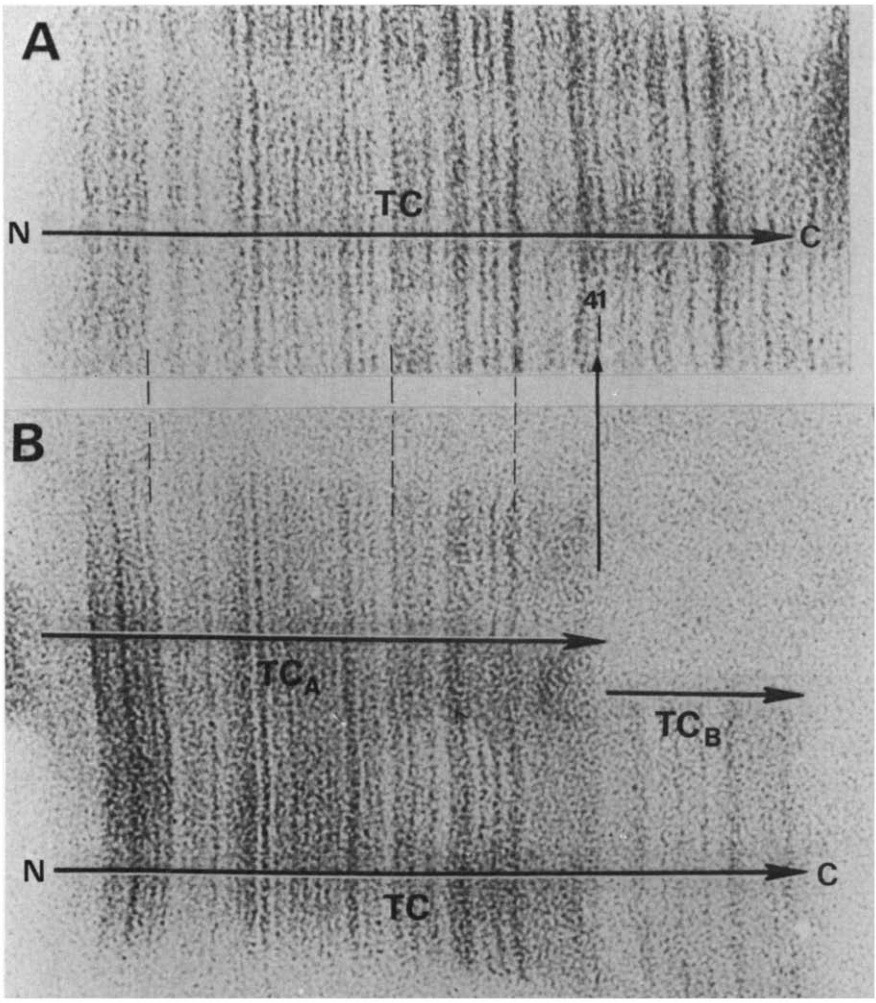

Fig. 3. Determination of the collagenase-mediated cleavage locus of the collagen molecule at 25°C from electron micrographs of segment-long-spacing crystallites of the reaction products. A. The TC segment-long-spacing crystallite has band 41 numbered according to the nomenclature of Bruns and Gross [17]. B. The TC_A crystallite is indicated and measures 75% of the length of the TC crystallite. Alignment of the banding patterns indicate the cleavage locus at interband 40.

The known collagenase inhibitors EDTA and 1.10 phenanthroline produced almost total inhibition, which suggests that metallic ions such as calcium and zinc [19] are essential for enzyme activity. The thiol reagents cysteine and dithiothreitol were also inhibitory, possibly through some chelation effect; but as *N*-ethylmaleimide was non-inhibitory it seems probable that thiol groups are not involved in collagenase activity. In contrast to an earlier report that mammalian corneal collagenase was inhibited by cartilage proteoglycans [17] the dog gingival enzyme was not affected by proteoglycan preparations derived from porcine or human cartilage. Whole dog serum, as expected, was shown to be a potent inhibitor of the gingival enzyme. (Table I).

To obtain a better understanding of the Beagle's serum inhibitors whole

TABLE I

EFFECT OF VARIOUS AGENTS AND SERUM PROTEINS ON GINGIVAL COLLAGENASE

The various agents were dissolved in 50 mM Tris · HCl buffer containing 0.17 M NaCl and 10 mM $CaCl_2$, adjusted to pH 8.0. 50 µl of dog gingival collagenase was used in each series of assays and control tubes contained 50 µl of tissue culture medium without enzyme. Each evaluation represents the mean of triplicate assays after subtraction of control values and is expressed as a percentage of the fibril degradation produced by the enzyme alone.

Reagent added	Concentration in assay	Enzyme activity cpm released	Inhibition (% of control)
None	—	1876	(0)
EDTA	10 mM	33	98
1,10-*o*-phenanthroline	1 mM	45	98
Cysteine	2 mM	784	58
	10 mM	239	87
Dithiothreitol	2 mM	308	84
	10 mM	0	100
N-ethylmaleimide	1 mM	1730	8
	10 mM	1940	0
None	—	1240	(0)
Proteoglycan, human	0.5 mg/ml	1190	4
Proteoglycan, porcine	0.5 mg/ml	1282	0
None	—	2180	(0)
Whole serum, human	2% (v/v)	51	98
Whole serum, beagle	2% (v/v)	0	100

serum was subjected to gel filtration studies on Sephadex G-200. Fig. 4 shows the resulting fractionation of serum proteins and the results of assaying the eluant fractions for inhibitory activity against collagenase and trypsin. Two regions of inhibition were observed for both enzymes. The inhibitor present in the first protein peak probably represents α_2-macroglobulin. This general antiproteinase has a molecular weight of approximately 725 000 and is eluted in the void volume of a Sephadex G-200 fractionation. The two enzymes showed

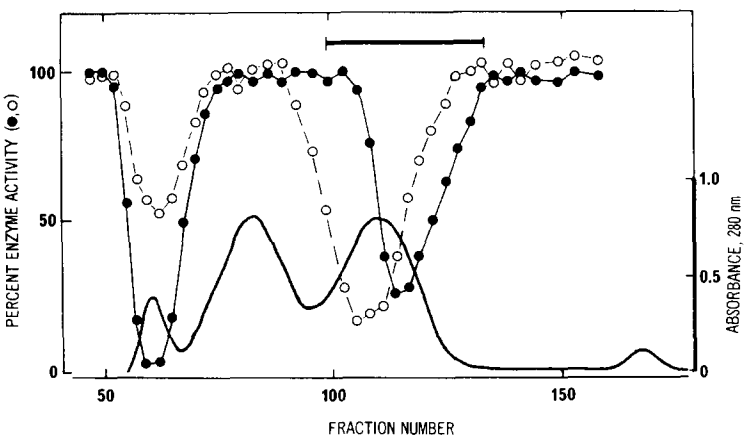

Fig. 4. Gel filtration of dog serum on Sephadex G-200 and inhibition of dog gingival collagenase. 2 ml dog serum was eluted from the column as described in Methods. Fractions were collected and each was examined for inhibitory activity against collagenase (●) and trypsin (○) assays, and plotted as a percentage of the control activity. The horizontal bar denotes the fractions pooled for concentration. ———, absorbance at 280 nm.

similar inhibitory profiles in this eluant region which suggests that they are both inhibited by the same antiproteinase. However, the inhibitory profiles for collagenase and trypsin in the elution region containing small molecular weight serum proteins are very different. The larger region of trypsin inhibition is probably explained by the presence of α_1-antitrypsin (fractions Nos. 90—120) whereas the region of collagenase inhibition represents a protein of slightly lower molecular weight (fractions Nos. 110—135). As these two inhibitory regions were not superimposed, suggesting two distinct inhibitors, the small molecular weight proteins were concentrated and applied to the ion exchange resin DEAE-Sephadex A.50.

The results of stepwise elution from the ion exchange resin is shown in Fig. 5 where the eluant fractions were examined for inhibitory activity in both collagenase and trypsin assays. Those fractions which inhibited the gingival collagenase had no effect on the trypsin activity, and those which produced trypsin inhibition had no effect on collagenase activity. The collagenase inhibitor was therefore separated from the trypsin inhibitor, probably α_1-antitrypsin, and those fractions containing the collagenase inhibitor were pooled and concentrated by ultrafiltration.

The concentrate was applied to a gel filtration column of Sephadex G-100 Superfine grade calibrated as described in Methods. The eluted fractions were assayed for collagenase inhibition and the position of the inhibitory activity corresponded to a molecular weight of approximately 40 000.

Characterization of the small collagenase inhibitor

Preparations of the 40 000-dalton collagenase inhibitor, after chromatographic separation, were examined by agarose electrophoresis. Fig. 6 shows the serum protein patterns of Beagle serum together with that of the

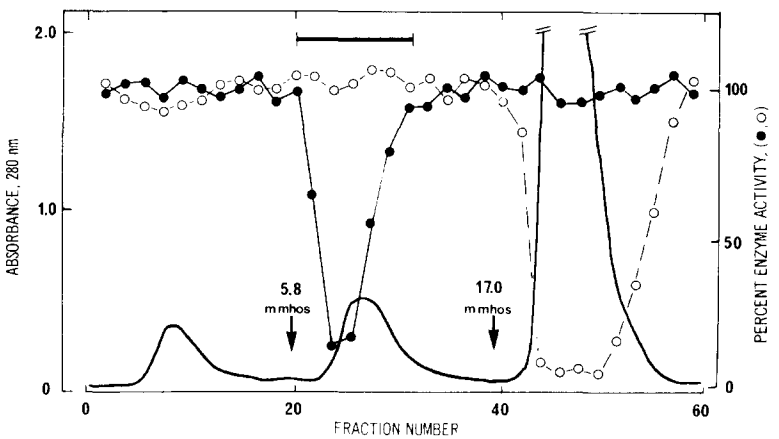

Fig. 5. Separation of the smaller collagenase inhibitor from the trypsin inhibitor by ion exchange chromatography on Sephadex DEAE, A-50. 2 ml of the concentrated protein solution obtained from Fig. 4 was applied to the resin and eluted with stepwise increases in NaCl concentration as shown. Each fraction was assayed for inhibitory activity against collagenase (●) and trypsin (○) which were plotted as percentages of the control activity. The horizontal bar denotes the fractions pooled for concentration. ———, absorbance at 280 nm.

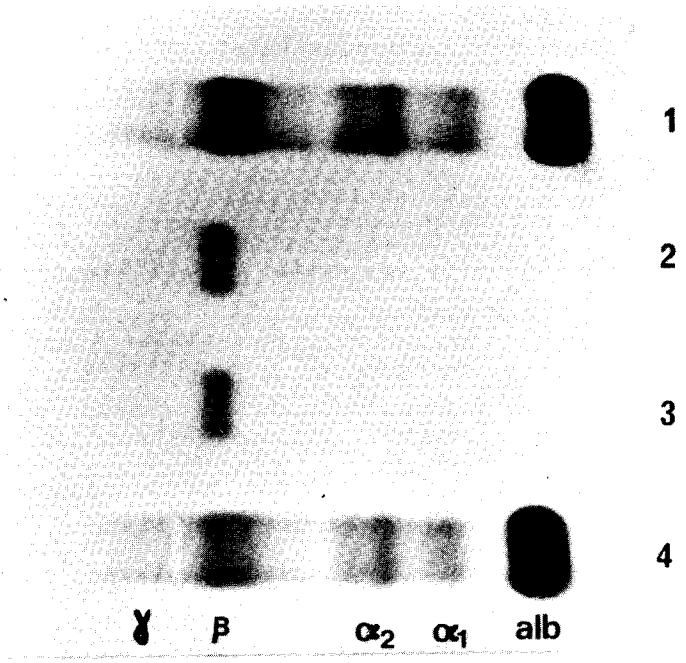

Fig. 6. Electrophoresis of the smaller collagenase inhibitor on Agarose. The partially purified inhibitor [2,3] and samples of whole dog serum [1,4] were applied to an agarose film and subjected to electrophoresis as described in Methods. The inhibitor behaved as a β-serum protein.

TABLE II

EFFECT OF THE β-SERUM INHIBITOR ON VARIOUS COLLAGENASES AND PROTEINASES

Inhibitory activity of the β-serum inhibitor was examined using the [^{14}C]collagen fibril assay with collagenases derived from various sources and adjusted to give similar activities. The collagenase activities are given as cpm released and each represents the mean of triplicate assays. The effect of the β-serum inhibitor on the proteinases trypsin, papain and thermolysin was examined using casein assays as described previously [11]. The absorbance values, measured at 280 nm for the trypsin and papain assays and 366 nm for the thermolysin assay, represent the mean of triplicate assays. Control incubations containing twice the amount of enzyme were included to ensure that reactions were terminated at a point where they were still linear. Figures in parentheses shown after the control enzyme activity indicate the proportion of substrate degraded. Assays were incubated for 16 h at 37°C, the collagenase assays contained 30 μg/ml of the β-serum inhibitor preparation and the proteinase assays contained 90 μg/ml.

Enzyme	Proteinase type	Substrate	Enzyme activity		% Inhibition
			Control	+inhibitor	
Collagenases					
Dog gingival	Metal	[^{14}C]collagen	2029	34	98
Human gastric mucosal	Metal	[^{14}C]collagen	2076	498	76
Human rheumatoid synovial	Metal	[^{14}C]collagen	1730	1070	38
Human skin	Metal	[^{14}C]collagen	2010	288	86
Guinea pig skin	Metal	[^{14}C]collagen	1524	564	63
Bacterial (*Clostridium histolyticum*)	Metal	[^{14}C]collagen	1906	2010	0
Proteinases					
Trypsin	Serine	Casein	0.140 (20%)	0.142	0
Papain	Thiol	Casein	0.09 (10%)	0.11	0
Thermolysin	Metal	Azocasein	1.240 (27%)	1.251	0

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF THE SERUM β-INHIBITOR AND α_2-MACROGLOBULIN ON DOG GINGIVAL COLLAGENASE

Inhibitory activity of the two dog serum antiproteinases was examined using the ^{14}C-collagen fibril assay. Each assay contained 50 µl of dog gingival collagenase and various amounts of each inhibitor. The final assay conditions were 0.17 M NaCl, 50 mM Tris-HCl buffer, pH 8.0, and 10 mM $CaCl_2$. The concentrations of the partially purified β-inhibitor preparation are given as total Lowry protein. The concentration of α_2-macroglobulin, obtained by 33% ammonium sulphate precipitation and gel filtration on Sephadex G-200, was determined immunologically by radial diffusion. Control assays contained equivalent amounts of each inhibitor inactivated by heating at 100°C for 5 min. Each value represents the mean of triplicate assays after subtraction of control values and is expressed as a percentage of the cpm released by the enzyme alone.

Serum antiproteinase	Concentration in assay (µg/ml)	Enzyme activity cpm released	Inhibition (% of control)
β-inhibitor	0	2029	(0)
	5	1562	23
	10	953	53
	20	630	70
	30	34	98
Heat-inactivated β-inhibitor	30	1988	2
α_2-macroglobulin	0	2007	(0)
	5	1605	20
	10	1264	37
	20	280	86
	25	40	98
Heat-inactivated α_2-macroglobulin	25	1966	2

small collagenase inhibitor. Electrophoretically the inhibitor behaved as a β-serum protein.

When this β-serum inhibitor of gingival collagenase was examined against other collagenases derived from human and guinea pig tissues, inhibition was observed in all cases but the extent of inhibition varied depending on the source of the enzyme. The bacterial collagenase of *Clostridium histolyticum* was not inhibited. (Table II)

When the β-inhibitor preparation was examined in trypsin, papain and thermolysin assays, enzymes chosen to represent the serine, thiol and metal-dependent proteinases, no evidence for inhibition was observed even when the concentration of inhibitor used in these assays was three times that required for complete inhibition of gingival collagenase (Table II). This suggests that the β-serum protein inhibitor of collagenase activity is not a general antiproteinase, but shows some degree of specificity.

The addition of increasing amounts of the β-inhibitor to constant amounts of gingival collagenase produced a linear response for enzyme inhibition as judged by the [^{14}C]collagen fibril assay. Similar results were obtained for the addition of α_2-macroglobulin preparations to the enzyme indicating that the degree of inhibition is proportional to the amount of inhibitor added. (Table III)

Discussion

In this paper we have shown that the collagenolytic activity released by dog gingival explants in culture has all the properties of a true collagenase. It is a Ca^{2+}-dependent enzyme, active at neutral pH and at 25° C attacks the collagen molecule at a single locus to produce two fragments, characteristic of collagenase action, representing 3/4 and 1/4 lengths of the molecule. The cleavage site was shown by SLS electron microscopy to be at interband 40 which is similar to the position found for other collagenases [18,20]. The molecular weight of the enzyme was estimated as 35,000 by gel filtration studies, and although this is similar to values obtained for human collagenases derived from rheumatoid synovium [21] gastric mucosa [10] and rabbit fibroblasts [22] it differs from values obtained for bovine gingival [23] and human gingival [24] enzymes.

The recent reports of latent forms of collagenase from gingival cultures [7—9] and the question as to whether or not these represent precursor forms of the enzyme or inhibitor-enzyme complexes, prompted us to examine in detail the collagenase inhibitors present in serum. As serum proteins are present in tissues when explanted but are probably washed out during culture, it would seem essential to identify and characterise those serum antiproteinases which react with collagenase in order to understand their relevance to in vitro studies. Moreover, the identification of natural inhibitiors is essential for our understanding of the in vivo role of the enzyme.

Fractionation of dog serum proteins by gel filtration showed that two regions of collagenase inhibition were present. One of these, the large molecular weight α_2-macroglobulin, is a recognised inhibitor of collagenases and usually accounted for approximately 60% of the collagenase inhibitory capacity of the whole serum. The small molecular weight inhibitor, shown to be a β-serum protein, accounted for approximately 40% of the inhibitory capacity of most dog serum samples. The contribution of the small molecular weight β-protein to the total serum inhibitory activity was much greater than that observed for human β_1-anticollagenase which probably constitutes only 5% of the total serum anticollagenase activity.

The β-serum protein inhibitor from dog serum was shown to have properties very similar to the β_1-anticollagenase protein of human serum. Both have molecular weights of approximately 40 000 and probably have similar protein charges as indicated by their mobility in agarose electrophoresis. The failure of the dog β-protein to inhibit three different proteinases indicates a high degree of specificity, as previously shown for β_1-anticollagenase [11]. Because of its small molecular size which would allow the β-inhibitor to permeate tissues from which α_2-macroglobulin would be excluded, it seems likely that the small collagenase inhibitor could have an important role in the extracellular regulation of collagenase activity in vivo.

Previous reports of serum inhibition of gingival collagenase showed that α_2-macroglobulin was the major inhibitor of the enzyme, but also suggested that α_1-antitrypsin was a less effective inhibitor of collagenase [25]. Our studies with purified α_1-antitrypsin have consistently failed to show any inhibition of dog gingival collagenase, or any other mammalian collagenase [11,26].

Reports of latent collagenase produced by cultures of gingival and other tissues are largely based on the activation of tissue culture media by mild trypsinization to release collagenolytic activity [8,9,27]. There has been much debate as to whether or not this activity results from the action of trypsin on a precursor or zymogen form of the enzyme or on inhibitor-enzyme complexes. The recent report that latent bone collagenase can be activated chemically with 4-amino-phenylmercuric acetate suggests that it represents an inhibitor-enzyme complex [28] and points to the importance of identifying and characterizing the natural inhibitors with which collagenase may react in vitro. In addition to the serum proteins α_2-macroglobulin and the β-inhibitor there is now some evidence that other collagenase inhibitors are synthesised or produced by tissues [28,29,30]. It seems essential that purified inhibitor-enzyme complexes should be examined in trypsinization and chemical activation experiments in order to obtain a better understanding of the concept of latent collagenase.

Microbial plaque extracts have been shown to activate latent gingival collagenase [31] suggesting an important relationship between dental plaque and collagenase in periodontal disease. Since plaque produces a variety of enzymes, endotoxins and antigenic substances, these factors could not only stimulate the production of collagenase by gingival cells, as demonstrated with endotoxin-stimulated macrophages [32], but might also activate some inhibited forms of the enzyme. Studies to elucidate the role and relationship of plaque and collagenase in periodontal disease are currently in progress.

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